New Series Vol. XVII No. 65 January 1953

Price 20*s*. net

ANNALS OF BOTANY

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OXFORD : AT THE CLARENDON PRESS
LONDON : GEOFFREY CUMBERLEGE

PRINTED IN GREAT BRITAIN
BY CHARLES BATEY AT THE UNIVERSITY PRESS, OXFORD

1953

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The Assimilation of Ammonia by Nitrogen-starved Cells of *Chlorella vulgaris*

Part I. The Correlation of Assimilation with Respiration

BY

P. J. SYRETT

(*Botany Department, University College, London*)

With fourteen Figures in the Text

ABSTRACT

The addition of ammonium sulphate to a suspension of nitrogen-starved *Chlorella* cells is followed immediately by the rapid assimilation of ammonia and a large increase of the respiration rate. The assimilation of ammonia and the high rate of respiration continue until either all the ammonia has been assimilated or some carbon reserve within the cells has been exhausted. Which happens first depends on the amount of ammonia added and the quantity of cells present. The respiration which accompanies ammonia assimilation is sensitive to cyanide and it has a respiratory quotient of 0·75 compared with 1·2–1·3 for normal respiration. The addition of glucose to nitrogen-starved cells when ammonia is being assimilated does not increase either the rate of respiration or the rate of assimilation. The rates of respiration and ammonia assimilation by normal cells are markedly increased by the addition of glucose. Light has little effect on the rate of ammonia assimilation by nitrogen-starved cells, but doubles the assimilation rate of normal cells.

INTRODUCTION

WHILE studying the respiration of the green alga *Chlorella vulgaris* it was found that the addition of ammonium nitrate to cells which had been allowed to photosynthesize for some time in a nitrogen-free medium was followed immediately by a *marked* increase in the rate of respiration. This effect was interesting enough to warrant further investigation. The stimulation of respiration was found to follow the addition of ammonium ions, and it was accompanied by a rapid assimilation of ammonia by the cells (see Fig. 4).

Cramer and Myers (1948) and Myers (1949) have also studied the effect of adding ammonium salts to nitrogen-deficient cells of a *Chlorella* species (*C. pyrenoidosa*). Their work, however, has been chiefly concerned with photosynthesis. Other work with nitrogen-deficient organisms has been done by White (1936), Roine (1947), McLean and Fisher (1947), and more recently by Willis (1951). This work will be discussed later in relation to the results presented here.

[Annals of Botany, N.S. Vol. XVII, No. 65, January, 1953.]

Material and Methods

Growth and treatment of cultures. The alga used was a strain of *Chlorella vulgaris*. It was grown in pure culture as previously described (Syrett, 1951). The same inoculation technique was used. The medium used to grow the cells contained 1·5 g. ammonium nitrate, 0·4 g. anhydrous magnesium sulphate, 0·4 g. sodium citrate, 3 drops saturated ferrous sulphate solution, 2 ml. of the A4 solution of Arnon (1938), together with 14·528 g. potassium dihydrogen phosphate and 4·646 dipotassium hydrogen phosphate to each 2 litres of 'Pyrex' distilled water. All the reagents used except the dipotassium hydrogen phosphate were of Analar quality. The pH of the medium, after autoclaving, was 6·0–6·1. When a nitrogen-free medium was required exactly the same medium was used with the omission of the ammonium nitrate.

The cultures were grown at 25° C. and illuminated by a tungsten filament lamp. The light intensity at the cultures was 600 foot-candles. They were aerated with a sterile stream of air containing 0·5 per cent. carbon dioxide. This was passed at the rate of 10 litres/hr. through each culture. When the cultures were 74–78 hours old they were harvested by centrifuging at 360*g* for 5 minutes. The cells were then washed twice with and resuspended in sterile nitrogen-free medium. The suspension was then transferred to culture bottles which were replaced in the culture tank. They were illuminated as before at 25° C. and air containing 0·5 per cent. carbon dioxide was passed through each bottle at the rate of 20 litres/hr.; 16–20 hours later the cells were centrifuged, washed once, and resuspended in nitrogen-free medium. As far as possible precautions were taken to prevent bacterial contamination of the cultures while doing this. None was ever observed.

When 'normal' cells were required for comparison with the nitrogen-deficient ones they were obtained by harvesting the cultures as usual after 74–78 hours. Instead of treating the cells with nitrogen-free medium, however, they were washed and resuspended in the full medium. Sixteen hours later they were centrifuged, washed, and resuspended in the nitrogen-free medium. A suspension of these 'normal' cells was dark green, while a suspension of nitrogen-starved ones was more yellow-green.

The measurement of respiration. Gas-exchange measurements were made by the Warburg manometric technique. The cells were suspended initially in nitrogen-free medium buffered at about pH 6·0. Two ml. cell suspension was pipetted into each flask. Ammonium sulphate was dissolved in the nitrogen-free medium and placed in the side-arm. Carbon dioxide was absorbed by 10 per cent. caustic potash in the centre well of the flask. When cyanide was used the caustic potash was replaced by the appropriate cyanide-potash mixture (Robbie, 1946). All experiments were carried out at 25° C. The bath was darkened by a cover of black plastic material.

The carbon dioxide production was measured by the direct method of Warburg (Dixon, 1943). A correction was applied for carbon dioxide retention in the medium (Umbreit, Burris, and Stauffer, 1949). To do this the pH

of the medium must be known accurately. The pH of the original suspending medium was measured electrometrically and so was that of the suspension in the flasks after the absorption of ammonia had occurred. As is to be expected, the absorption of ammonia is accompanied by a decrease of the pH of the medium. Owing to the buffering capacity of the medium, however, the change is not very great; after the absorption of 12 micromoles ammonia from ammonium sulphate the change in pH was only 0·14 units. The error introduced by this change into the correction factor for carbon dioxide retention is very small.

When fermentation was measured the manometers were filled with oxygen-free nitrogen and the carbon dioxide production followed.

The measurement of ammonia assimilation. Unless otherwise stated, 20 ml. of a suspension of cells in nitrogen-free medium was pipetted into a 100-ml. conical flask. This was shaken in the same darkened bath and at the same time as the Warburg flasks in which respiration was followed. At a known time a certain quantity of ammonium sulphate dissolved in nitrogen-free medium was added to the suspension. One-millilitre samples were withdrawn at intervals and pipetted into the outer chambers of Conway units (Conway, 1947). To these 1 ml. of saturated potassium carbonate solution was added. This killed the cells and liberated ammonia present in both the cells and the medium. The ammonia was absorbed by a boric acid solution placed in the central chamber of the unit; after the units had stood for 2 hours at room temperature it was titrated with N/100 sulphuric acid.

Preliminary experiments showed that the amount of ammonia present in either 'normal' or nitrogen-deficient cells before the addition of ammonium sulphate was too small to be detected by the method used.

Results

The stimulation of the rate of respiration by the addition of ammonium salts

The effect of adding ammonium nitrate, ammonium sulphate, potassium nitrate, and potassium sulphate on the oxygen uptake of nitrogen-starved cells is shown in Fig. 1. The rate of oxygen absorption is greatly stimulated by the addition of ammonium salts; potassium nitrate produces less stimulation. Ammonium sulphate is just as effective as ammonium nitrate, which suggests that the effects of ammonia and nitrate are not additive. The rate of carbon dioxide production is also increased (Fig. 3).

The increased respiration rate is inhibited by cyanide (Fig. 2). Thus, in this respect, the high respiration rate is similar to that which follows the addition of glucose to *Chlorella* cells (Emerson, 1927; Syrett, 1951).

The fermentation rate of nitrogen-starved cells is low and is only slightly increased by the addition of ammonium sulphate (Fig. 3); the presence of glucose makes no difference.

The assimilation of ammonia. When ammonium sulphate is added to nitrogen-starved cells the ammonia is rapidly assimilated. The assimilation is

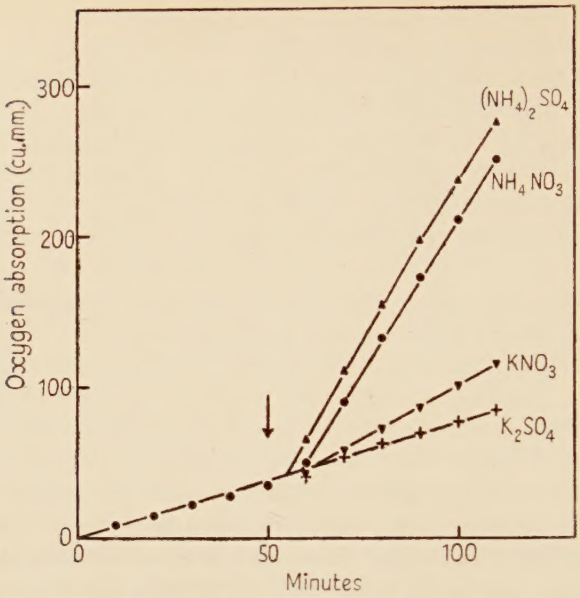

FIG. 1. The effect of various salts on the oxygen absorption of nitrogen-starved cells. Each salt was added, at the time indicated by the arrow, to give a final concentration of M/100. (17·35 mg. dry wt. cells in each flask; pH 6·1.)

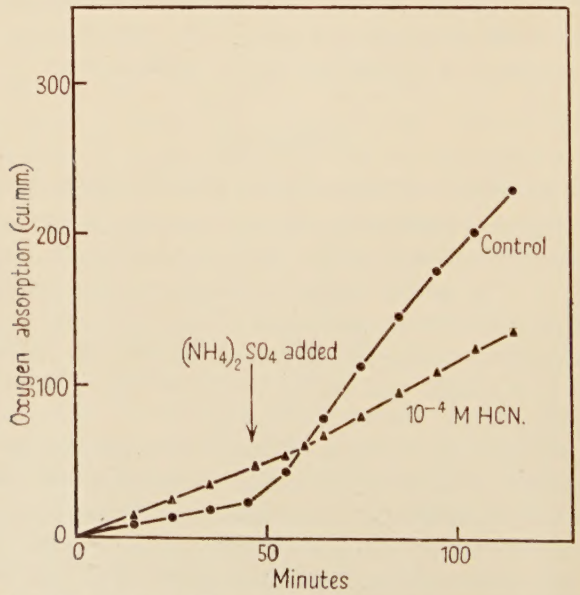

FIG. 2. The effect of cyanide on the oxygen absorption of nitrogen-starved cells. 10 micromoles ammonium sulphate were added at the time indicated. (14·0 mg. dry wt. cells in each flask; pH 6·2.)

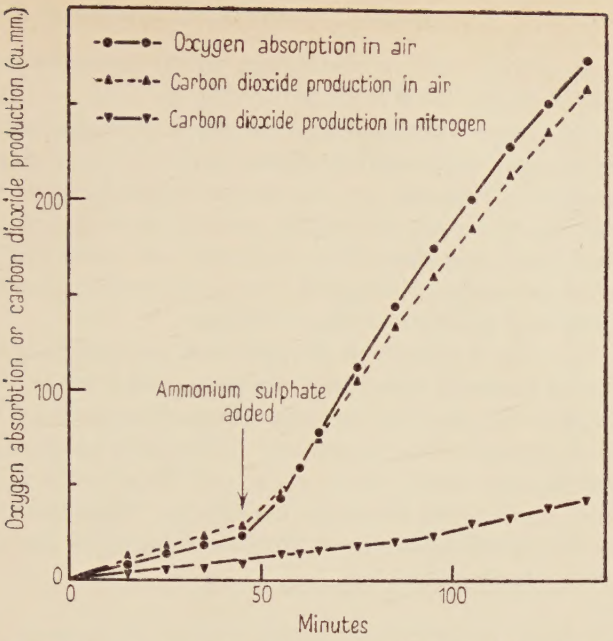

FIG. 3. The effect of ammonium sulphate on the respiration and fermentation of nitrogen-starved cells. 10 micromoles ammonium sulphate were added to each flask at the time indicated. The fermentation curve is the same whether 1 per cent. glucose is present or not. (14·0 mg. dry wt. cells in each flask; pH 6·2.)

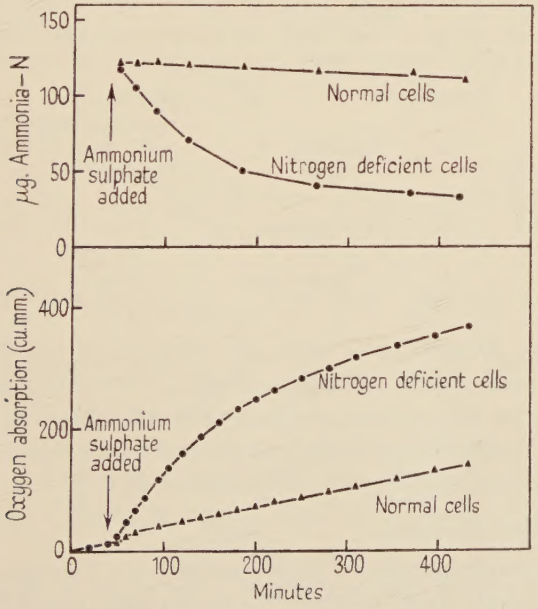

FIG. 4. The correlation of ammonia assimilation with oxygen absorption. The upper curves show ammonia assimilation by normal and nitrogen-starved cells. The lower curves were obtained at the same time with samples from the same stock suspensions of cells. 5 micromoles ammonium sulphate per ml. suspension were added at the time indicated. (The suspension contained 4·04 mg. dry wt. cells/ml.; pH 6·1.)

accompanied by a high rate of respiration, which decreases as the rate of assimilation slows down. This is shown by Fig. 4.

The volume of oxygen which had been absorbed when any ammonia sample was taken can be easily obtained from these curves. If this is done the rate of ammonia assimilation between two successive sampling times can be compared with the rate of oxygen absorption over the same period. The figures so obtained with the nitrogen-starved suspension are plotted in Fig. 5. It is clear that the rate of oxygen absorption is directly proportional to the rate of ammonia assimilation throughout the experiment.

It appears, too, that if conditions are such that respiration is little affected by the addition of ammonia then little assimilation takes place. The contents of the flasks which were used for the experiments illustrated by Figs. 2 and 3 were analysed at the end of the experiment. The results are shown in Table I; much less assimilation takes place when 10^{-4} M cyanide is present and practically none at all under anaerobic conditions. Thus the stimulation of respiration by the added ammonia and the assimilation of that ammonia are presumably very closely connected.

TABLE I

The effect of Cyanide and Anaerobic Conditions on Ammonia Assimilation by Nitrogen-starved Cells

Each flask contained 14·0 mg. dry wt. cells. 20 micromoles ammonia (as ammonium sulphate) were added to each flask. pH 6·21.

The ammonia remaining in each flask was determined 190 minutes after it was added. Each figure is the mean of determinations on two identical flasks.

Gas phase.	Additions to flask.	Ammonia remaining in each flask (micromoles).	Ammonia assimilated (micromoles).
Air	—	0	20·0
Air	1% glucose	0	20·0
Nitrogen	—	19·3	0·7
Nitrogen	1% glucose	19·4	0·6
Air	10^{-4} HCN	15·1	4·9

'Normal' cells, in contrast to nitrogen-starved ones, assimilate the added ammonia much more slowly. A slight stimulation of the rate of oxygen uptake follows the addition of ammonia to these cells, but this is of short duration. It is presumably connected with the short period of nitrogen starvation which the cells undergo before the ammonium salt is added.

The effect of added glucose

The rate of oxygen uptake of both 'normal' and nitrogen-starved cells is increased by the addition of glucose (Table II; Figs. 6 and 7). The effect is more marked with the 'normal' cells. When ammonium sulphate is added to the cells as well as glucose the rate of oxygen uptake is again increased. This effect is more marked with the nitrogen-starved cells. It is interesting to note

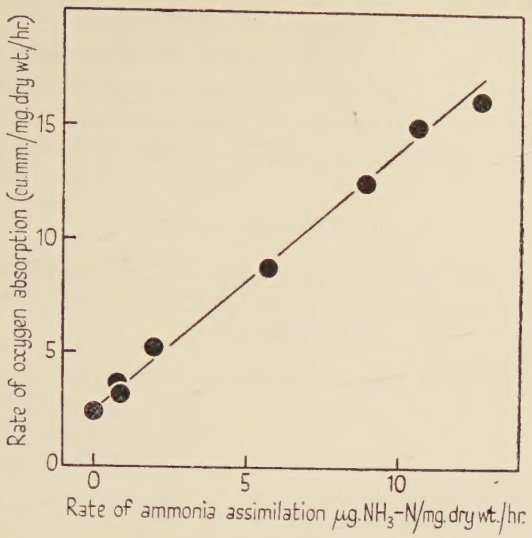

FIG. 5. The results of Fig. 4 plotted to show the relationship between the rate of oxygen absorption and the rate of ammonia assimilation of nitrogen-starved cells.

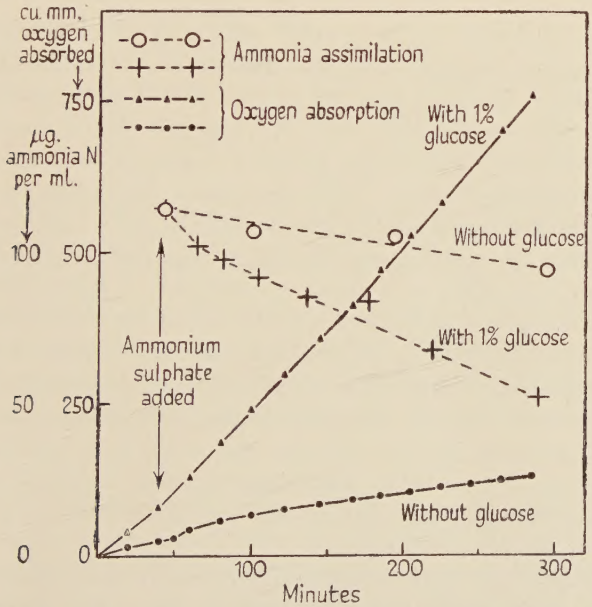

FIG. 6. The effect of glucose on the oxygen absorption and ammonia assimilation of 'normal' cells. 5 micromoles ammonium sulphate per ml. suspension were added at the time indicated. (The suspension contained 4·75 mg. dry wt. cells/ml.; pH 6·1.)

that after the addition of ammonium sulphate the rate of oxygen uptake of the nitrogen-starved cells with or without glucose present is much the same as that of the 'normal' cells with glucose (Table II). This suggests that the cells under these different conditions are respiring at a rate limited by some common factor. This rate of oxygen uptake is much the same as that of cells which have suffered no nitrogen starvation whatever and which are respiring glucose.

TABLE II

The Effect of Ammonium Sulphate on the Rate of Oxygen Absorption by Nitrogen-starved and 'Normal' Cells in the Presence and Absence of Added Glucose

Rates given as cu. mm. oxygen per mg. dry wt. cells per hour.

		Before addition of ammonium sulphate.	After addition of ammonium sulphate.
Nitrogen-starved cells	With no added glucose . .	2·74	16·08
	With 1% glucose present . .	7·75	16·66
'Normal' cells	With no added glucose . .	3·05	8·52 (for 10 minutes only)
			2·32
	With 1% glucose present . .	12·48	17·53

The addition of glucose to the 'normal' cells markedly increases the rate of ammonia assimilation just as it increases the rate of respiration (Fig. 6). The rate of assimilation, however, is never as high as that of the nitrogen-starved cells. The addition of glucose to these starved cells clearly does not increase their rate of ammonia assimilation, just as it does not increase their rate of respiration appreciably (Fig. 7). Fig. 7 also shows that the rate of oxygen uptake decreases to about its former rate when all the added ammonia has been assimilated. This also takes place whether or not added glucose is present.

The effect of cell quantity on ammonia assimilation. A comparison of Figs. 4 and 7 shows that whereas in the first experiment all the ammonia added to the cells was not assimilated (Fig. 4), in the second experiment it was (Fig. 7). Now the same quantity of ammonia was added per unit volume cell suspension in both experiments, but the cell suspension used for the first experiment contained only 4·04 mg. dry wt. cells per ml., while that used for the second contained 7·65 mg. dry wt. cells. This suggested that the amount of ammonia that a given quantity of cells could rapidly assimilate under these conditions was limited.

This was confirmed by another experiment. From a suspension of nitrogen-starved cells less concentrated suspensions were prepared by dilution. Two ml. of each suspension was pipetted into Warburg flasks. Ten micromoles ammonium sulphate, dissolved in nitrogen-free medium, were placed in each side-arm. The flasks were attached to their manometers and oxygen absorption measured. On tipping the ammonium sulphate into the cell sus-

pensions the rate of oxygen uptake increased markedly and the high rate continued for some time. When it was certain that the rapid rate of oxygen uptake had ceased in a flask it was removed and a 1-ml. sample taken for an ammonia determination. The flask was then replaced on its manometer and shaken for a further 30 minutes. Then another 1-ml. sample was taken. The difference in the ammonia content of the two samples was small. It gives, however, a measure of the rate of the slow ammonia uptake which occurs after

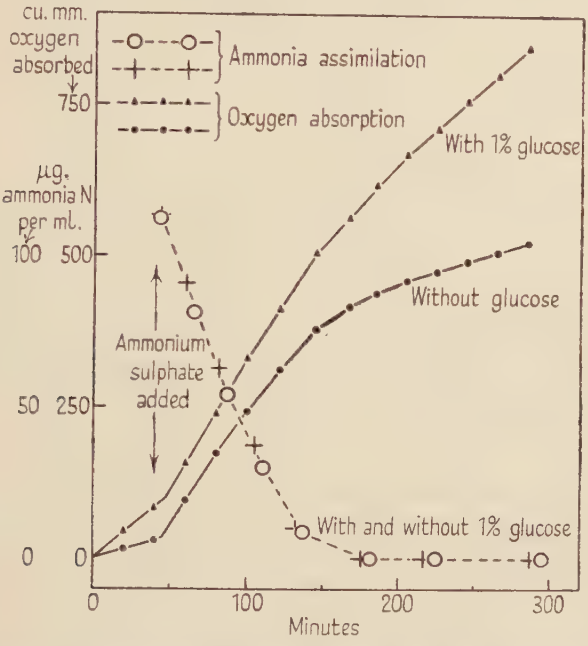

FIG. 7. The effect of glucose on the oxygen absorption and ammonia assimilation of nitrogen-starved cells. 5 micromoles ammonium sulphate per ml. suspension were added at the time indicated. (The suspension contained 7·65 mg. dry wt. cells/ml.; pH 6·1.)

rapid assimilation has ceased (Fig. 4). This was used to apply a small correction to the figures because some time elapsed between the cessation of rapid oxygen absorption and sampling for the first time. This correction was only necessary with the more dilute cell suspensions which did not utilize all the ammonia added.

The result of this experiment is shown in Fig. 8.

When a flask contained a cell dry weight of 13 mg. or more all the ammonia added was rapidly assimilated. If it contained less, the amount of ammonia used was proportional to the quantity of cells present. Thus a given number of cells could assimilate only a limited amount of ammonia. It seems likely that this was due to the depletion of some carbon substrates within the cells. When 13 mg. or more cells were present the total amount of carbon substrates in them was sufficient to allow all the added ammonia to be assimilated. When fewer cells were present ammonia assimilation could continue only as long as

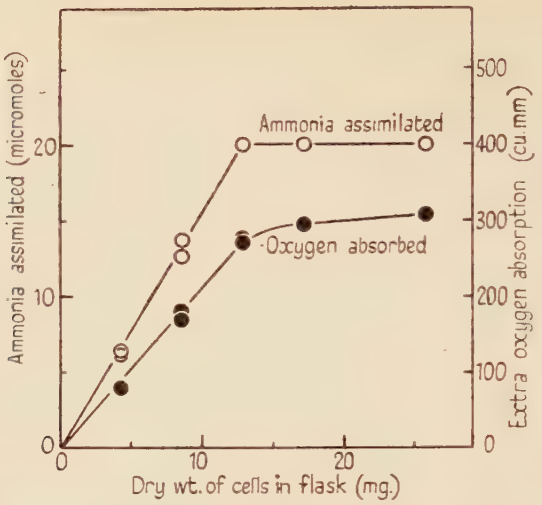

FIG. 8. The effect of cell quantity on the assimilation of ammonia by nitrogen-starved cells. Along the abscissa is plotted the dry weight of the cells present in each Warburg flask. To each flask 20 micromoles of ammonia (as ammonium sulphate) were added. The ordinate shows the amount of this ammonia which was assimilated in each flask. Also plotted is the volume of oxygen absorbed during the assimilation of the ammonia. This volume was estimated by method 3 (see text and Fig. 10). It makes little difference to the form of this curve if another method is employed.

the carbon reserves lasted. That this is so is indicated by Table III, which shows the effect of adding glucose to a small quantity of cells assimilating ammonia. In the absence of glucose, assimilation stops when about two-thirds of the added ammonia has been used. When glucose is present it is all used, just as it is when more cells are there. Thus it appears that glucose can supplement the carbon reserve within the cells.

TABLE III

The Effect of the Addition of Glucose on the Assimilation of Ammonia by a Small Quantity of Cells

Contents of flask.	Ammonia remaining when assimilation ceased (micromoles).	Ammonia assimilated (micromoles).
8·6 mg. dry wt. cells	7·4	12·6
8·6 mg. dry wt. cells	6·3	13·7
8·6 mg. dry wt. cells + 20 mg. glucose .	0	20·0
8·6 mg. dry wt. cells + 20 mg. glucose .	0	20·0
12·9 mg. dry wt. cells	0	20·0
12·9 mg. dry wt. cells	0	20·0

Fig. 8 also shows the volume of oxygen which was absorbed in each flask during the metabolism of the added ammonia; it is roughly proportional to the amount of ammonia which has been assimilated.

The volume of oxygen absorbed during the assimilation of a given quantity of ammonia

In order to investigate this four experiments were carried out. For each a suspension containing about 7 mg. dry wt. nitrogen-starved cells per ml. was prepared. Such a suspension assimilated completely all the ammonia added during an experiment. Two ml. of suspension was placed in each of a series of Warburg flasks. Different quantities of ammonium sulphate, containing from 4 to 12 micromoles ammonia, were added from the side-arms. The

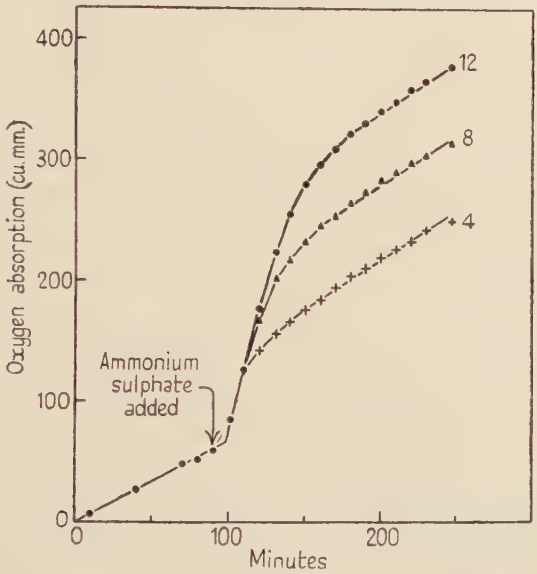

FIG. 9. The effect of various quantities of ammonia on the oxygen absorption of nitrogen-starved cells. Ammonium sulphate was added at the time shown. The figures by the curves show the number of micromoles ammonia which were added.

oxygen absorption was followed. The result of a typical experiment is shown in Fig. 9. As expected, the volume of oxygen absorbed increases with the quantity of ammonia added.

When one attempts to calculate exactly the volume absorbed per micromole of added ammonia one is immediately faced with the difficulty of deciding whether the basic oxygen uptake continues during the period of ammonia assimilation or not. Further, if one decides one way or the other about this it is still not clear how the volume of oxygen is best calculated from the graphs. At least five different methods can be used to calculate this volume from the graph of oxygen uptake against time. These are shown diagrammatically in Fig. 10.

When the ammonia is added the rate of oxygen uptake increases immediately (Fig. 7). Thus the transition point *A* is quite clear. The rate of oxygen absorption, however, tails off rather slowly, but an examination of Fig. 7 shows that

not until point *B* is reached has all the ammonia disappeared. Consequently, if one assumes that while any ammonia is still present all the oxygen uptake is associated with ammonia assimilation, the difference between *A* and *B* must be calculated. This is method 2 in Fig. 10. It may be, however, that only as long as the rate of oxygen uptake is very rapid is all of it associated with ammonia assimilation; when the rate decreases both the basic respiration and the extra 'ammonia respiration' may go on together. If this is so, method 1 is probably the best way of estimating the volume of oxygen taken up. Both these methods assume that the rapid oxygen uptake which follows the addition of ammonia

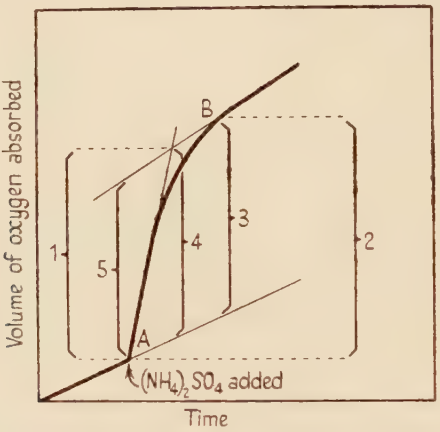

FIG. 10. Diagram to illustrate five possible methods of estimating the volume of oxygen absorbed during the metabolism of a given quantity of ammonia. The thick line represents the actual oxygen absorption curve which is obtained (cf. Fig. 9). The thin lines represent linear extensions of parts of this curve. For a further discussion of this, see text.

is all connected with ammonia assimilation. If, on the other hand, the basic respiration is assumed to continue quite independently of the ammonia metabolism, three other methods of calculation can be used. Method 3 assumes that the basic respiration continues at the same rate until all the ammonia has been assimilated. This is unlikely since the rate of respiration after the ammonia has all gone is invariably slightly higher than it was before it was added. Method 5 assumes that the rate of basic respiration during the assimilation period is the same as it is after assimilation is complete. Method 4 is a compromise between methods 3 and 5.

In Table IV the results of the four experiments carried out are summarized. The volume of oxygen has been calculated by each of the five methods described here. By comparing the standard deviations of the means some estimate of the variability of the results can be made. It can be seen that the results are most variable when calculated according to methods 2 and 3. This does not mean that these methods are less satisfactory theoretically than the other methods. The results are more variable because it is more difficult to determine point *B* with certainty than the other points.

TABLE IV

The Volume of Oxygen absorbed during the Assimilation of a Given Quantity of Ammonia

The mean values for a number of experiments are given together with the standard errors of the means.

Micromoles ammonia added (as $(NH_4)_2SO_4$).	Number of experiments.	Volume of oxygen absorbed (cu. mm.) estimated by five different methods (see text and Fig. 10)				
		1.	2.	3.	4.	5.
4	4	78·00 ±2·38	90·25 ±4·81	69·25 ±2·49	66·75 ±2·76	62·25 ±3·25
6	3	112·67 ±1·46	131·67 ±4·85	102·33 ±2·06	96·67 ±1·34	90·33 ±3·12
8	4	150·00 ±2·04	172·25 ±3·70	138·00 ±1·36	129·75 ±1·55	120·00 ±2·27
10	4	186·50 ±1·93	212·00 ±5·07	170·75 ±2·14	163·50 ±1·19	153·75 ±1·11
12	4	219·25 ±3·09	244·75 ±4·15	197·00 ±4·14	189·50 ±3·01	177·75 ±2·94
Vol. of oxygen absorbed per micromole ammonia added. (Estimated from graph of volume against ammonia added (Fig. 11).)		17·82	19·47	16·20	15·62	14·72
Atoms of oxygen consumed per molecule of ammonia added		1·59	1·74	1·45	1·40	1·32

When the volume of oxygen absorbed is plotted against the quantity of ammonia added the points lie on a straight line. This line should pass through the origin, and it almost does so no matter which method of calculation has been used. It is farthest out when method 2 is used. This indicates that this method slightly over-estimates the volume of oxygen taken up. One cannot say, however, from this whether one method is theoretically better than the rest. Fig. 11 shows the lines obtained when the volumes calculated by method 2 and 5 are plotted. The other methods of calculation give lines which lie between these two, but they are omitted for clarity.

The slope of the line fitted to the points gives the volume of oxygen taken up per micromole of ammonia added. These values are given in Table IV. They vary from 14·7 to 19·5 cu. mm., depending on the method of calculation which has been used.

In order to write a stoichiometric equation connecting ammonia assimilation with oxygen uptake one would like to know how much oxygen is taken up during the metabolism of 1 micromole ammonia. It can be seen that it is very difficult to find this with certainty as one cannot say which of the five figures shown in Table IV is the best estimate of this value.

On the whole it seems unlikely that all the respiration which follows the addition of ammonia is connected with the metabolism of the added ammonia.

This idea receives some support from two observations. Firstly, if the whole of the respiration which follows the addition of ammonia were connected with ammonia assimilation, one would not expect a straight-line relationship between the rate of oxygen absorption and the rate of ammonia assimilation (Fig. 5). Rather one would expect a curve which was concave towards the abscissa. The simplest interpretation of the straight-line relationship is that the 'ammonia respiration' is superimposed on a constant basic respiration. Secondly, support comes from the experiment graphed in Fig. 7. Here

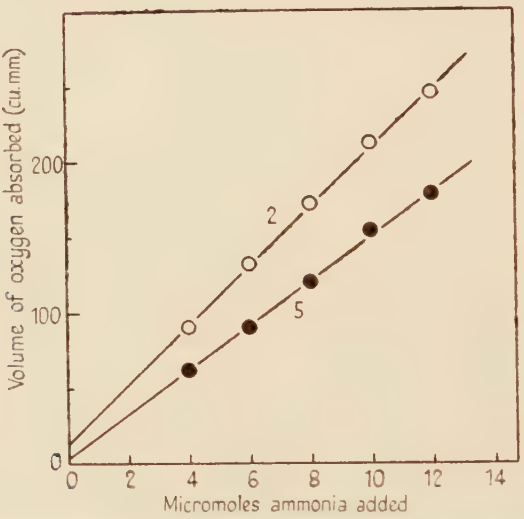

FIG. 11. The mean volume of oxygen absorbed during the metabolism of a certain quantity of ammonia is shown here. Each point is the mean of 3 or 4 experiments (see Table III). The standard error of each mean is less than the radius of the points as they are plotted. The lines drawn are those which best fit the observations. They were calculated by the method described by Fisher (1946). The upper line joins the values estimated by method 2, the lower those obtained by method 5 (see text and Fig. 9). The values obtained by the other methods lie between these.

10 micromoles of ammonium sulphate were added to the same quantity of cells with and without added glucose. Before the ammonium sulphate is added the rate of respiration is about three times higher in the presence of 1 per cent. glucose. After the addition of ammonia the rate of respiration is the same whether glucose is present or not. The graph of ammonia absorption against time (Fig. 7) shows that all the added ammonia is assimilated in both flasks and that it is assimilated at the same rate. Under these conditions it seems reasonable to expect that the oxygen absorption connected with the ammonia metabolism will be the same both with and without added glucose. The volume of oxygen absorbed, calculated by the five different methods described here, is shown in Table V. By no method of calculation, however, is the volume of oxygen absorbed the same whether glucose is present or not. When methods 1 and 2 are used the volume absorbed is apparently much

larger when glucose is present than when it is not; when the other three methods are used the volume is apparently smaller. The first two methods assume that the basic respiration ceases during the ammonia metabolism, the last three that it continues. Neither assumption is probably quite correct. If one can divide the respiration of a living cell into clear-cut parts—which seems unlikely—what happens here is that the basic respiration is modified to some extent as soon as the ammonia is added. The results in Table V suggest that to assume that the basic respiration continues is probably nearer to the truth than to assume that it ceases.

TABLE V

The Volume of Oxygen absorbed during the Assimilation of 20 micromoles Ammonia

Volumes given as cu. mm. oxygen absorbed

	Method of estimation of volume absorbed (see text and Fig. 10)				
	1.	2.	3.	4.	5.
A. Glucose absent . . .	353	406	311	294	269
B. 1% glucose present . .	459	525	251	248	215
Difference (B–A) . .	+106	+119	−40	−46	−54

Consequently, all one can say about the volume of oxygen absorbed when ammonia is added is that it is between 14·7 and 19·5 cu. mm. per micromole added ammonia according to the assumptions one has to make in calculating it. The lower figure is probably nearer the truth than the upper one. This uncertainty means that one cannot write a stoichiometric equation connecting ammonia assimilation and oxygen absorption. In view of the varied fate of the ammonia added, however, it is doubtful whether any such equation would mean very much.

The carbon dioxide production during ammonia assimilation by nitrogen-starved cells

Several experiments were carried out in which both the oxygen absorption and the carbon dioxide production were measured. The results of two typical experiments are shown in Figs. 3 and 12.

The rate of both processes increases markedly when ammonium sulphate is added. The rate of carbon dioxide production, however, does not increase as much as the rate of oxygen uptake. Consequently, the respiratory quotient falls immediately after the addition of ammonia. This is shown in Fig. 12 and also in Table VI. The value of the respiratory quotient is lowest, 10–20 minutes, after the addition of ammonium sulphate. The higher value during the 10-minute period immediately following the addition of ammonia is presumably due to the 90-second interval which elapsed between reading any manometer and adding the ammonium salt. How long the lowered respiratory

quotient lasts depends on the amount of ammonium sulphate added. It begins to rise again before the rapid rate of oxygen uptake ceases, i.e. before all the ammonia added is assimilated.

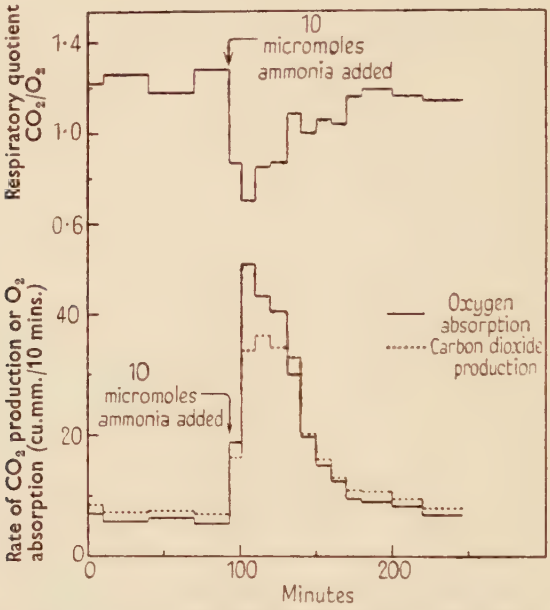

FIG. 12. The effect of ammonia on the rates of oxygen absorption and carbon dioxide production by nitrogen-starved cells. The upper curve shows the effect on the respiratory quotient. 10 micromoles ammonia (as ammonium sulphate) were added at the time indicated. (14·7 mg. dry wt. cells in each flask; pH 6·15.)

The lowest value of the respiratory quotient which was measured was 0·70. Usually it was about 0·75 (see Table VI). This can only be taken as a true value of the respiratory quotient for ammonia metabolism if all the respiration during this period is connected with ammonia assimilation. If, on the other

TABLE VI

The Effect of Ammonium Sulphate on the Respiratory Quotient (R.Q.) of Nitrogen-starved Cells

Each value is the mean value from eight experiments. The standard error of each mean is given.

R.Q. before addition of ammonium sulphate	1·290 ±0·024
R.Q. during 10-minute period immediately following addition of ammonium sulphate	0·831 ±0·015
R.Q. during period 10–20 minutes after addition of ammonium sulphate	0·749 ±0·009
R.Q. after the rapid rate of oxygen uptake has ceased	1·165 ±0·009

hand, when ammonia is added the increased respiration is simply superimposed on a basic respiratory system, the true respiratory quotient of the 'ammonia respiration' will be somewhat lower. The effect of this is best illustrated by the analysis of one typical experiment. The figures are given in Table VII. It can be seen that the respiratory quotient of the extra 'ammonia respiration' is 0·68 if the basic respiration is assumed to continue unaltered while the ammonia is assimilated.

TABLE VII

The Estimation of the Respiratory Quotient (R.Q.) of the Extra 'Ammonia Respiration' if it is assumed that a Basic Respiratory System continues to operate while Ammonia is being assimilated

The 'basic' rate is taken as the mean of the rate before ammonia is added and the rate when assimilation has ceased.

All rates are given as cu. mm. gas absorbed or evolved per flask per hour.

	Oxygen uptake.	Carbon dioxide output.	R.Q.
Rates of gas exchange during 1 hour period before addition of ammonium salt	35·0	45·5	1·30
Rates of gas exchange when the assimilation of ammonia has ceased	40·5	47·0	1·16
Mean values ('basic' rates)	37·75	46·25	1·23
Rates of total gas exchange during period 10–20 minutes after addition of ammonium salt	290·0	217·0	0·75
Rates of gas exchange during the period allowing for the basic rates as calculated above . .	252·25	170·75	0·68

The effect of light on ammonia assimilation

The results of an experiment carried out to investigate this are shown in Fig. 13. Suspensions of normal and nitrogen-starved cells were prepared in nitrogen-free medium. One portion of each type of cell suspension was shaken in a darkened thermostat tank, another was shaken in a similar tank which was illuminated from below. The temperature of both tanks was exactly the same. The light intensity at the level of the suspensions was 280 foot-candles. At a certain time ammonium sulphate was added to each suspension and the assimilation of ammonia was followed by sampling at intervals.

The results plotted in Fig. 13 show that, in darkness, the rate of assimilation by normal cells is quite slow, but that it is approximately doubled by illumination. On the other hand, the rate of assimilation by nitrogen-deficient cells is very fast and illumination only slightly increases it.

A further experiment with nitrogen-starved cells was made to investigate the relationship between ammonia assimilation and illumination in the presence and absence of added glucose. The results of this experiment are shown in Fig. 14. The suspension was not dense enough to assimilate all the

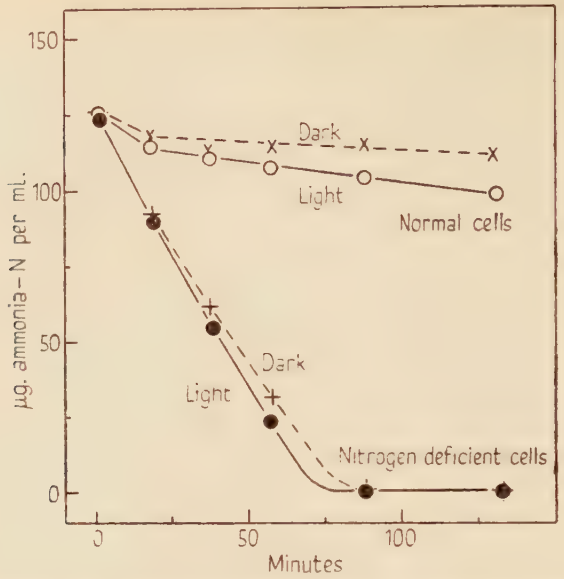

FIG. 13. The effect of light on ammonia assimilation by normal and nitrogen-starved cells. 5 micromoles ammonium sulphate per ml. suspension were added at zero time. Nitrogen-starved suspension contained 7·54 mg. dry wt. cells/ml. 'Normal' suspension contained 7·69 mg. dry wt. cells/ml. (pH 6·1; temperature 25·5° C.; light intensity 280 foot-candles.)

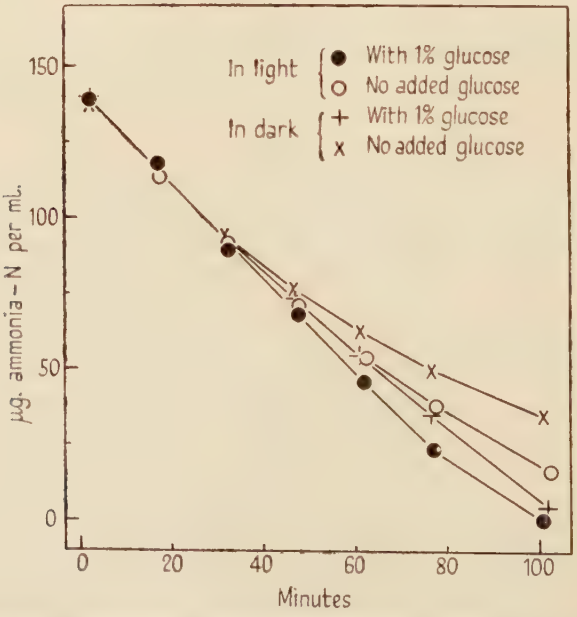

FIG. 14. The effect of glucose and light on the assimilation of ammonia by nitrogen-starved cells. 5 micromoles ammonium sulphate per ml. suspension were added at zero time. (The suspension contained 6·98 mg. dry wt. cells/ml.; pH 6·1; temperature 25·5° C.; light intensity 280 foot-candles.)

added ammonia in the absence of any added carbon source (cf. Fig. 4). In darkness and without any added glucose the rate of assimilation soon decreased. When glucose is present or the cultures are illuminated more assimilation occurs. Immediately after the addition of the ammonium salt, however, the rate of assimilation is much the same under all conditions. Presumably this is because the cells contain sufficient carbohydrate to saturate the enzyme systems involved.

These results will be discussed together with those presented in Part II of this paper.

The Assimilation of Ammonia by Nitrogen-starved Cells of *Chlorella vulgaris*

Part II. The Assimilation of Ammonia to other Compounds

BY

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With three Figures in the Text

Abstract

Ammonia is rapidly assimilated by nitrogen-starved *Chlorella* cells and converted into soluble organic nitrogenous compounds. During the first 20 minutes of assimilation most of the ammonia which has been used can be accounted for as free or combined α-amino-nitrogen and amide-nitrogen. Later a smaller proportion of the assimilated ammonia is found in these fractions while appreciable quantities of basic amino-acids are formed. The assimilation of ammonia is accompanied by an increase of the rates of oxygen absorption and carbon dioxide production; the value of the respiratory quotient decreases. Non-reducing sugar and acid-hydrolysable polysaccharide are metabolized rapidly.

Possible interpretations of these facts are discussed.

Introduction

In Part I of this communication it was shown that nitrogen-starved cells of *Chlorella vulgaris* assimilate ammonia very quickly. The assimilation is accompanied by a marked increase in the rate of respiration. Before possible reasons for this increase can be discussed more knowledge of what happens to the assimilated ammonia is needed. This was investigated by the experiments described here.

Material and Methods

The growth and treatment of the cells is described in Part I. The methods used to measure respiration are also described there.

The estimation of the nitrogenous fractions

In each experiment the total nitrogen per ml. suspension was determined as ammonia after the Kjeldahl digestion of two aliquots of the suspension. The insoluble nitrogen of any sample was taken as the difference between the total nitrogen present in that volume and the soluble nitrogen extracted from it.

[Annals of Botany, N.S. Vol. XVII, No. 65, January, 1953.]

The extraction of soluble nitrogenous compounds from the cells

The method used to extract these was one which caused little breakdown of glutamine (see discussion of methods by Steward and Street, 1946). Ten ml. cell suspension was pipetted into a centrifuge tube containing 0·5 ml. 2·2 per cent. trichloracetic acid. This lowered the pH to 5·6. The tube was heated in a boiling-water bath for 1½ minutes in order to kill the cells. It was then kept at 80° C. for 10 minutes. The cells were removed by centrifuging (5 minutes at 480*g*) and the clear extract was poured into a 25-ml. volumetric flask. The cells were then extracted for 15 minutes at 80° C. with 5 ml. 0·1 per cent. trichloracetic acid. They were then centrifuged, the extract decanted, and the cells extracted once more in the same way. The combined extracts were made up to 25 ml. with distilled water. The extract, with a little toluene added, was stored at 0° C.

Preliminary experiments showed that this treatment removed most of the soluble nitrogen from the cells. When one sample of cells was extracted five times in this way 78 per cent. of the nitrogen extracted was present in the first extract, 10·5 per cent. in the second, and 7 per cent. in the third; the fourth and fifth together contained only 4·5 per cent. Thus over 95 per cent. of the nitrogen extracted was present in the first three extracts.

The estimation of the nitrogen fractions in the extract

All determinations were done twice. If the two determinations differed by more than 1 μg. nitrogen they were repeated.

(*a*) *Total soluble nitrogen.* This was determined as ammonia after micro-kjeldahl digestion. 0·1 ml. digestion mixture was added to 0·5 ml. extract in a 150×10 mm. Pyrex tube which was covered by a loose-fitting glass cap. The extract contained no nitrate so that no preliminary reduction of this was necessary. Digestion was continued for at least 8 hours. The digestion mixture used was 10N sulphuric acid containing 0·2 per cent. sodium selenate and 0·2 per cent. copper sulphate pentahydrate.

When digestion was finished the contents of the tube were carefully washed into the outer chamber of a Conway unit (Conway, 1947). Ammonia was liberated by adding 1 ml. 40 per cent. caustic potash to the outer chamber and absorbed by N/20 sulphuric acid in the central chamber of the unit. The unit was allowed to stand overnight. The acid from the central chamber was then transferred to a graduated tube; water and 2 ml. Nessler reagent (Folin and Denis, 1916) were then added and the mixture was made up to 10 ml. After the tube had stood for 15 minutes the colour intensity was measured in a photo-electric colorimeter using a filter transmitting light of wave-length 410–460 mμ. The colour intensity was compared with that of freshly prepared ammonium sulphate standards.

(*b*) *Ammonia nitrogen.* 0·5 ml. extract was pipetted into the outer chamber of a Conway unit. The ammonia was liberated by the addition of 1 ml. saturated potassium carbonate and absorbed in N/20 sulphuric acid. The

units were allowed to stand for 2 hours at room temperature. The ammonia absorbed by the acid was then determined as described above.

(*c*) *Amide nitrogen.* Total amide nitrogen was determined by the method described by Conway (1947). 0·5 ml. extract was digested for 3 hours at 100° C. with 0·25 ml. 3N sulphuric acid. After digestion the ammonia was determined and compared with that originally present. The increase of ammonia nitrogen was taken as total amide nitrogen.

Unstable amide nitrogen (glutamine) was determined by the method of Vickery et al. (1935). The difference between this and the total amide nitrogen was taken as stable amide nitrogen (asparagine).

(*d*) *Free amino-nitrogen.* This was determined by the method of Frame, Russell, and Wilhelmi (1943) as modified by Russell (1944). All volumes and reagent concentrations were altered, however, so that the final volume was 10 ml. instead of 15 ml. Standards were run with each set of determinations and these received exactly the same treatment as the samples. The standard solution was an equimolecular mixture of alanine and glutamic acid containing 30 μg. amino-nitrogen per ml. Each standard also contained the same quantities of trichloracetic acid and other salts as each sample. The small quantity of trichloracetic acid present did not appear to interfere with colour development.

Free ammonia gives some colour with the reagents and must therefore be removed. To do this the sample of extract was boiled for 2 minutes *in vacuo* with normal caustic soda. The alkali was then carefully neutralized and the determination of amino-nitrogen carried out. The intensity of the colour developed was read in a photo-electric colorimeter using a filter transmitting light of wave-length 450–500 mμ.

This method was found to be very satisfactory. After the final addition of reagents the colour changed rather quickly during the first 10 minutes, but then it was perfectly stable for at least 1 hour at room temperature. Consequently, the tubes were allowed to stand for about 15 minutes before measuring the colour intensity.

Preliminary experiments showed that 96–100 per cent. of alanine and glutamic acid amino-nitrogen added to samples was estimated by this method. The amino-nitrogen content of one sample was kindly determined by Dr. L. Fowden using a colorimetric ninhydrin method (Moore and Stein, 1948). It was found to agree within 4 per cent. with the amount determined by the usual method.

(*e*) *Combined amino-nitrogen.* This was determined from the increase in amino-nitrogen after the hydrolysis of a portion of the extract with an equal volume of concentrated hydrochloric acid. The mixture was heated in a sealed tube for 24 hours at 105° C. No humin was formed. The acid was removed by drying *in vacuo* over caustic soda, the residue dissolved in water, and amino-nitrogen determined after the removal of ammonia as described above.

Standards were run with each set of determinations; each standard received exactly the same treatment as the samples.

The estimation of the carbohydrate fractions

Two ml. of cell suspension was pipetted into a 15-ml. centrifuge tube. The cells were spun down by centrifuging at 480*g* for 5 minutes. The supernatant liquid was poured away and the tube heated for 5 minutes in a boiling-water bath. The cells were then resuspended in 5 ml. 70 per cent. alcohol and allowed to stand for 72 hours at 25° C. The cells were then centrifuged and the extract decanted. Two further extractions of the cells with 70 per cent. alcohol, each for 24 hours, were made. The combined extracts were made up to 25 ml. with water and stored at 0° C. Soluble carbohydrates in this extract were estimated.

(*a*) *Reducing sugar*. The Hagedorn and Jensen (1923) method as modified by Fujita and Iwatake (1931) was used to estimate the reducing value of the extract. The results were expressed as mg. equivalent glucose. Two ml. extract were taken for each determination. Replicate determinations agreed to within 3 per cent.

(*b*) *Non-reducing sugar*. This was determined from the increase in reducing value of the extract after acid hydrolysis and was expressed as mg. equivalent glucose. Two ml. extract were hydrolysed with 0·4N hydrochloric acid for 10 minutes at 100° C.; the acid was neutralized and reducing value determined.

(*c*) *The determination of insoluble acid-hydrolysable polysaccharide*. Two ml. 3 per cent. V/V sulphuric acid was added to the cell residue remaining after the extraction of the soluble carbohydrates. The tube was then heated for 3 hours at 100° C. to hydrolyse polysaccharides. The acid was neutralized and the cell remains extracted with hot water. The extract was made up to 25 ml. One ml. samples were taken for a determination of reducing value.

Experiments

A suspension of nitrogen-starved cells was prepared containing about 7 mg. dry wt. cells/ml. Aliquots of this were pipetted into two 250-ml. conical flasks. These were shaken in a darkened thermostat tank at 25° C. At a suitable known time sufficient ammonium sulphate dissolved in nitrogen-free medium was added to one of the flasks to give an ammonia concentration of 10 micromoles/ml. An equivalent volume of nitrogen-free medium was added to the other flask. After these additions samples were withdrawn at intervals from both flasks: 10-ml. samples for the determination of the nitrogenous fractions and 2-ml. ones for the carbohydrate estimations.

While this was being done the respiration of 2-ml. samples of the same suspension was followed in Warburg manometers. Equivalent quantities of ammonium sulphate solution or nitrogen-free medium were tipped into the Warburg flasks at about the same time as these solutions were added to the bulk of the suspension in the 250-ml. conical flasks. Both the oxygen uptake and carbon dioxide output were measured—in duplicate—on the suspension to which ammonium sulphate was added, singly on the control suspension. The replicate determinations agreed to within 1–2 per cent.

Two such experiments were carried out at different times. The results of the first are shown in Fig. 1, of the second in Fig. 2.

These Figures show that the addition of ammonium sulphate to nitrogen-starved cells is followed by the rapid assimilation of the ammonia added. This is accompanied by a large increase in the rate of respiration and it can be seen that the rate of oxygen uptake increases more than the rate of carbon dioxide production, i.e. the respiratory quotient decreases (see Fig. 3).

As the ammonia is assimilated the other soluble nitrogenous compounds in the cells increase. After 3 hours, 75 per cent. of the ammonia added in expt. 1, and 80 per cent. in expt. 2, is present in the cells in a soluble form. The remainder has been converted into insoluble compounds, presumably chiefly protein. When the increase in insoluble nitrogen is plotted it can be seen that little increase occurs in the first 20 minutes after the addition of the ammonium salt.

The increase in the soluble nitrogen is due to an increase in several fractions. During the first 30 minutes the quantity of amide-nitrogen present increases quite sharply and then remains at a fairly constant level. A third experiment has been carried out in which the amide-nitrogen of both asparagine and glutamine has been determined separately (Table I). An increase of both amides occurs, but more glutamine is formed than asparagine. There is also a steady increase of both free and combined amino-nitrogen.

TABLE I

The Synthesis of Amides by Nitrogen-starved Chlorella Cells

Stable amide-nitrogen (asparagine amide-nitrogen) and unstable amide-nitrogen (glutamine amide-nitrogen) were determined. The results are expressed as μg. N/ml. suspension. 128 μg. ammonia-N was added at zero time.
The suspension contained 9·3 mg. dry wt. cells/ml.; pH 6·1.

Time of sampling (min.)	Ammonia-N μg.	Total amide-N μg.	Stable amide-N μg.	Unstable amide-N μg.
0	128·0	3·3	1·4	1·9
9	104·7	13·2	6·1	7·1
14	99·5	19·8	4·6	14·8
24	73·0	19·8	7·1	12·7
45	39·8	25·4	6·6	18·8

The changes in the carbohydrate fractions are also shown in Figs. 1 and 2. As soon as ammonia is added a rapid decrease of the acid-hydrolysable polysaccharide fraction occurs. There is also a decrease in the disaccharide fraction, but little change of free reducing sugar occurs.

DISCUSSION

The formation of organic nitrogenous compounds

When ammonium sulphate is added to nitrogen-starved cells of *Chlorella vulgaris*, ammonia is rapidly assimilated and organic nitrogen compounds are

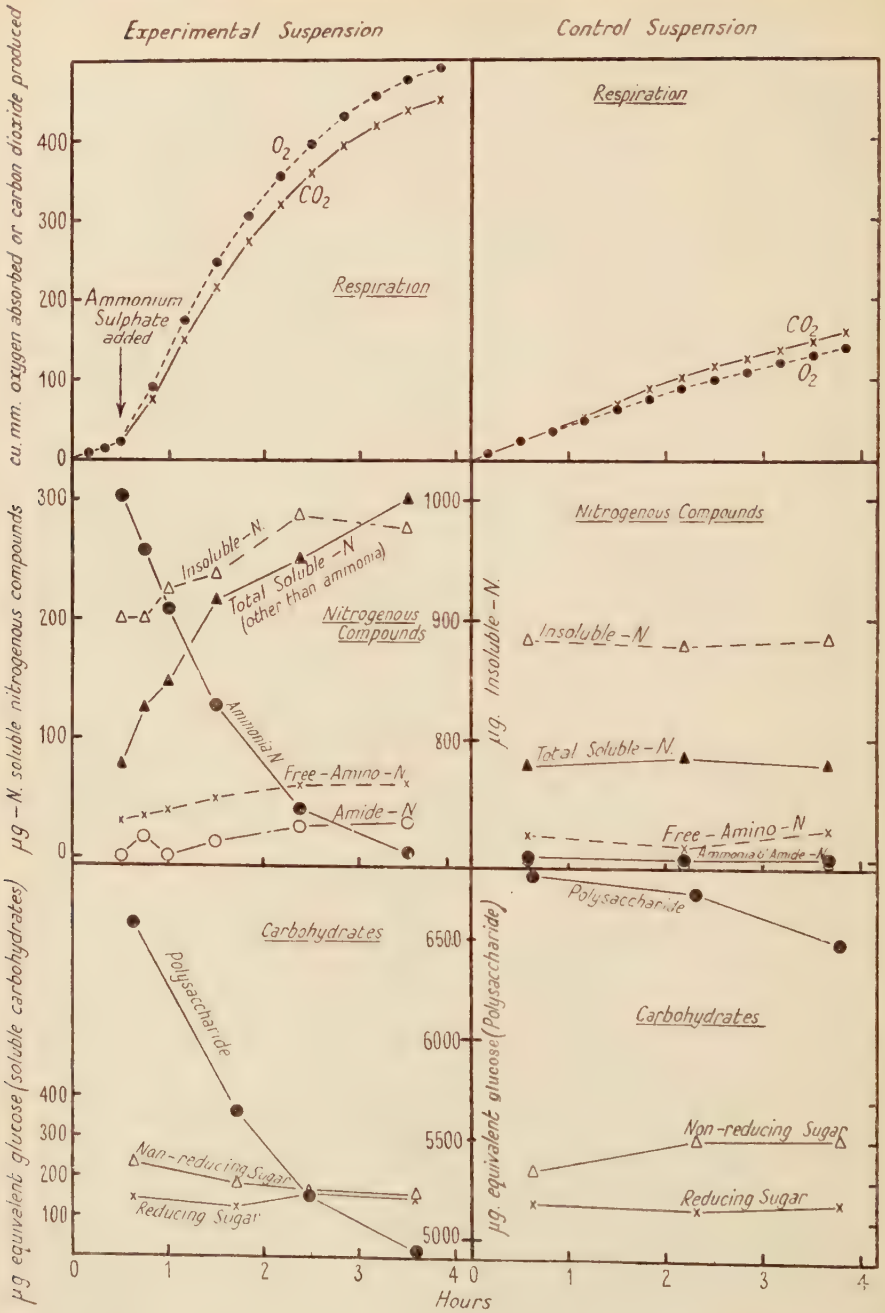

FIG. 1

FIGS. 1 and 2. The effect of the addition of ammonium sulphate on the respiration, nitrogen, and carbohydrate fractions of nitrogen-starved cells. After 30 minutes sufficient ammonium sulphate solution was added to the experimental flasks to give an initial ammonia concentration of 10 micromoles/ml. At the same time an equivalent volume of nitrogen-free medium was added to the control flasks.

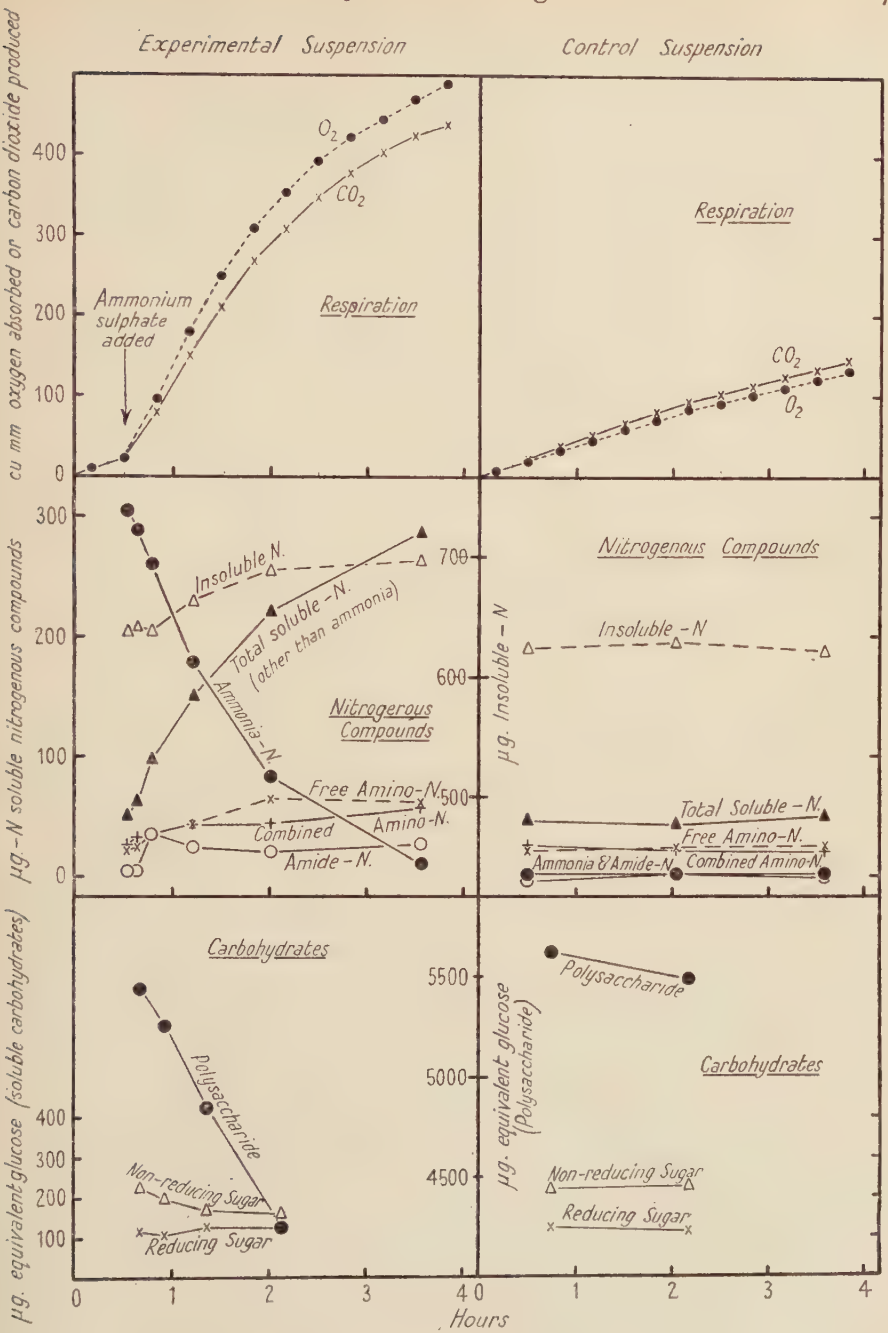

FIG. 2

The results are expressed as cu. mm. gas, μg.-nitrogen or μg. equivalent glucose per 2·0 ml. suspension before the addition of ammonium sulphate solution or nitrogen-free medium.

Fig. 1 shows the results of expt. 1 and Fig. 2 those of expt. 2.

Expt. 1. Initial pH of medium = 6·1; dry wt. of cell suspension (before additions) = 8·40 mg./ml. Expt. 2. Initial pH of medium = 6·1; dry wt. of cell suspension (before additions) = 7·24 mg./ml.

formed. During the first 30 minutes of assimilation the amide-nitrogen fraction increases most. This has also been found by workers with other organisms. Roine (1947), using nitrogen-starved yeast, found that practically all the ammonia assimilated in the first 20 minutes after its addition could be accounted for as amide, amino-dicarboxylic acid, or alanine nitrogen. The greatest increase was in the total nitrogen of the amide fraction; this was chiefly glutamine. Willis (1951) has reported that nitrogen-starved barley

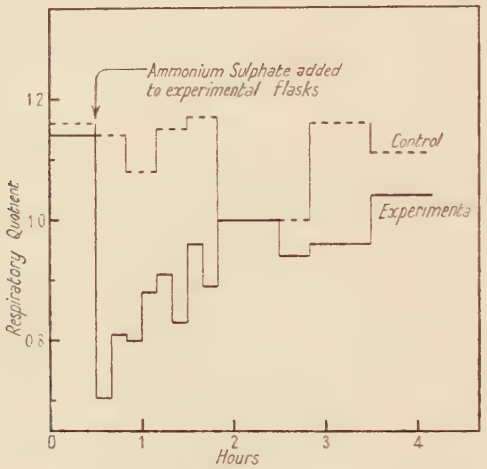

FIG. 3. The data of expt. 2 (see Fig. 2) plotted to show the effect of the addition of ammonium sulphate on the respiratory quotient. After 30 minutes ammonium sulphate solution was added to the experimental flask and nitrogen-free medium to the control flask.

roots assimilate ammonia rapidly. In these, too, the nitrogen compound which increases most at first appears to be glutamine. In *Chlorella* both asparagine and glutamine are formed; but while the formation of asparagine soon ceases, that of glutamine continues until 70 per cent. of the amide present is glutamine (Table I). It appears that amides, which are of great importance in the nitrogen metabolism of other organisms, play a similar role in *Chlorella*.

After the initial increase of the amides no further increase of this fraction occurs. The increase in soluble nitrogen after this is due to an increase of both the free and combined amino-nitrogen (Figs. 1 and 2). Roine (1947) also found an increase of free amino-nitrogen; this was almost entirely due to the formation of amino-dicarboxylic acids and alanine. He did not attempt to determine the increase in combined amino-nitrogen. Little can have been present in his extracts, however, as the fractions he estimated accounted for nearly all the soluble nitrogen present. The composition of this combined amino-nitrogen fraction is uncertain. It is possible that such a fraction would not have been extracted from yeast cells by Roine since he extracted them with 8 per cent. trichloracetic acid. The *Chlorella* extracts were made with very dilute acid (0·1 per cent.) which precipitates less protein (Steward and

Street, 1946). If this is so, any increase in this fraction would have been estimated by Roine as an increase of insoluble nitrogen.

Nevertheless, in *Chlorella* this combined amino-nitrogen fraction differs from the insoluble nitrogen fraction which is not extracted by hot 0·1 per cent. acid. Three hours after the addition of ammonia the combined amino-nitrogen has increased by 140 per cent.; the insoluble nitrogen has increased by only 9·5 per cent. At the beginning of the experiment the combined amino-nitrogen is 4 per cent. of the insoluble nitrogen; at the end it is 8 per cent. This is difficult to explain merely on the assumption that the combined amino-nitrogen represents protein nitrogen soluble under the extraction conditions used here. There seems little doubt that as assimilation proceeds both the free amino-acids and also some soluble combinations of them increase together. Attempts to precipitate the combined amino-nitrogen with trichloracetic acid and tungstic acid failed. The nature of this fraction must therefore be left undecided at present.

TABLE II

A Comparison of the Soluble Nitrogen Fractions determined with the Total Soluble Nitrogen

Results from expt. 2. The figures represent μg. nitrogen.

Time of sampling in minutes from the addition of ammonia	2	8	17	42	91	184
Total soluble N	51·2	62·5	98·5	150·7	220·2	285·8
Fractions determined						
Amide-N	4·1	4·5	34·6	23·8	20·4	27·2
Free amino-N	22·7	25·6	33·6	44·4	64·3	62·5
Combined amino-N	25·6	32·4	34·7	43·7	44·4	60·2
Total	52·4	62·5	102·9	111·9	129·1	149·9
Percentage of the total soluble N accounted for by these fractions	102·5	100·0	104·5	74·2	58·6	52·5

When the sum of the estimations of the various nitrogen fractions is compared with the estimation of the total soluble nitrogen an interesting discrepancy can be seen (Table II). For 20 minutes after the addition of the ammonia these fractions account for all the soluble nitrogen present. After this their importance diminishes, and after 3 hours' assimilation they account for only 52 per cent. of the soluble nitrogen. It was suggested by Dr. L. Fowden that the synthesis of appreciable quantities of the basic amino-acids might account for this discrepancy since these would contain nitrogen other than the α-amino nitrogen which was determined. To see whether this was so he kindly made filter-paper chromatograms of some of the hydrolysed extracts. When these were developed with ninhydrin it was clear that after ammonia assimilation has gone on for 40 minutes there is a large increase not only of alanine, glutamic, and aspartic acids, as Roine (1947) found with yeast, but also of the basic amino-acids. These were chiefly arginine and another which may be ornithine or lysine. A comparison of chromatograms of

hydrolysed and unhydrolysed extracts showed that a large increase of glutamic acid occurred on hydrolysis; this was presumably formed from glutamine. Some increase of arginine also occurred. The basic amino-acids synthesized appear to be present largely in the free form. Whether the amount of basic amino-acids synthesized will account for the discrepancy in the soluble nitrogen fractions can only be determined by quantitative analysis. The basic amino-acids, however, are certainly an important part of the soluble nitrogenous compounds in the cell.

The formation of appreciable quantities of basic amino-acids helps to explain another point. From the decrease in the carbohydrate fractions it is possible to calculate how much carbohydrate carbon is available for the synthesis of nitrogenous compounds. It is assumed that 180 g. of carbohydrate, estimated as 'equivalent glucose', contains 6 g.-atoms of carbon. An allowance is made for carbon lost as carbon dioxide. When this is done the amount of carbohydrate carbon which was available for synthesis can be compared with the amount of assimilated nitrogen. Two such C/N ratios are calculated in Table III. In both experiments the value of this ratio is just over 2. This is a remarkably low value since few organic nitrogen compounds which are found in plants have a C/N ratio as low as this. The C/N ratio of glutamine, for example, is 2·5; for glutamic acid and most other amino-acids it is even higher. One of the compounds with a lower value is arginine; its C/N ratio is 1·5. Consequently the formation of appreciable quantities of this would produce a low overall value of the C/N ratio. It is probable, however, that compounds other than carbohydrate are used for the synthesis of nitrogenous compounds. The organic acids, for example, must almost certainly be involved. If this is so the C/N ratios calculated here will be lower than the overall C/N ratio of the nitrogenous compounds which are synthesized.

The fact that it is the acid-hydrolysable carbohydrate fractions which decrease during ammonia assimilation agrees well with other work. When glucose is assimilated by *Chlorella* most of it is converted into acid-hydrolysable carbohydrates (Syrett, 1951). Thus these carbohydrates appear to constitute a carbohydrate reserve. They are formed either in darkness from glucose or in light from carbon dioxide. They are used when conditions are such that carbohydrate is metabolized quickly, as in these experiments.

If carbohydrate is being respired rapidly during ammonia assimilation, the low value of the respiratory quotient must be due to the conversion of some of the carbohydrate to more oxidized compounds. Since amino-acids and amides are synthesized at this time it seems likely that much of the carbohydrate is oxidized to keto-acids which are used for this synthesis. Such a conversion would partly account for the low value of the respiratory quotient.

The effect of added ammonium sulphate on the rate of respiration

The addition of ammonium sulphate to nitrogen-starved cells is followed at once by a large increase in the rates of both oxygen consumption and carbon dioxide production. The respiration rate is then as high as is that of normal

cells in the presence of glucose (Part I, Table I), giving what appears to be the maximum rate which is attainable by *Chlorella*. This suggests that the respiratory enzyme systems become saturated with substrate as soon as ammonia is added.

TABLE III

An Estimate of the Carbon and Nitrogen available for the Synthesis of Organic Compounds

All figures refer to 2 ml. of the original cell suspension (i.e. 2·266 ml. after the addition of ammonium sulphate). Carbohydrate given as μg. equivalent glucose; carbon dioxide in cu. mm.; ammonia as μg. nitrogen

Experiment I

Time of sampling.	Reducing sugar.	Disaccharide.	Acid-hydrolysable poly-saccharide.	Carbon dioxide production.	Ammonia.
8 min.	297	469	6567	44·5	280·0
186 min.	*306*	*326*	*4936*	*433·0*	*4·5*
Amount consumed	−9	+143	+1631	−388·5	+275·5
		+1765			
Expressed as μg.-atoms carbon or nitrogen		+58·83 C		−17·35 C	19·7 N
Total carbon and nitrogen available for synthesis			41·5 μg.-atoms C		19·7 μg.-atoms N

$$\text{C/N ratio} = \frac{41{\cdot}5}{19{\cdot}7} = 2{\cdot}11$$

Experiment II

Time of sampling.	Reducing sugar.	Disaccharide.	Acid-hydrolysable poly-saccharide.	Carbon dioxide production.	Ammonia.
10 min.	238	458	5435	44·0	281·0
91 min.	*254*	*323*	*4321*	*291·0*	*83·6*
Amount consumed	−16	+135	+1114	−247·0	+197·4
		+1233			
Expressed as μg.-atoms carbon or nitrogen		+41·1 C		−11·0 C	+14·1 N
Total carbon and nitrogen available for synthesis			30·1 μg.-atoms C		14·1 μg.-atoms N

$$\text{C/N ratio} = \frac{30{\cdot}1}{14{\cdot}1} = 2{\cdot}14$$

Although this increase of respiration rate is accompanied by a rapid decrease in the carbohydrate present in the cells, it is not certain that carbohydrate is the respiratory substrate. However, the respiration of nitrogen-starved cells to which no ammonia has been added (Figs. 1 and 2) and that of normal cells (Syrett, 1951) is accompanied by a decrease in acid-hydrolysable polysaccharide and this is sufficient to account for the carbon dioxide production. Here, then, carbohydrate appears to be the respiratory substrate, and it is probable that the same is true of nitrogen-starved cells which are assimilating ammonia. Whatever the source of the carbon dioxide, however, one has to explain how the addition of ammonia produces such a marked and immediate change in the rate of respiration.

An increase of the rate of respiration of plant tissue after the addition of ammonium salts or nitrates has been observed several times (Hamner, 1936; Sankaran, 1936; White and Templeman, 1937; McLean and Fisher, 1947, 1949; Said and El-Shishiny, 1948; Willis, 1951). The increase is especially marked if the tissue is nitrogen-starved. Myers (1949) has shown that a sharp change in the value of the respiratory quotient occurs when ammonia is added to nitrogen-deficient cells of *Chlorella pyrenoidosa*. Most of the previous workers, however, have used rather bulky tissues and, probably because of this, the rise of the respiration rate after the addition of ammonium salts is not as rapid as it is with *Chlorella*. An exception to this is the work of McLean and Fisher (1947, 1949) with the bacterium *Serratia marcescens*. They found that the rate of respiration of resting cells was increased as soon as ammonia was added. This initial rapid increase was followed by a later and slower increase which was connected with the growth of the cells.

One general conclusion which can be drawn from these studies is that the addition of ammonium salts or nitrates to a nitrogen-starved tissue will be followed by an immediate increase in the rate of respiration provided that some carbon compounds are available for metabolism. These may either be endogenous carbohydrates (as in the *Lemna* plants studied by White and Templeman, 1937) or added externally (as in the work of McLean and Fisher with *Serratia*, 1947, 1949).

Possible explanations of the stimulation of respiration by ammonium salts

Any explanation of the effect of added ammonia on the respiration of nitrogen-starved *Chlorella* cells must explain the following facts:

1. The respiration rate of nitrogen-starved cells is low.
2. It increases as soon as ammonium sulphate is added; the high rate continues only for as long as ammonia is being assimilated (Part I, Figs. 4, 7, and 9).
3. Glucose increases the respiration rate of nitrogen-starved cells, but the rate is further increased by the addition of ammonium sulphate; the rate then reached is much the same as if ammonia alone had been added (Part I, Table I).

4. The respiratory quotient of nitrogen-starved cells immediately after the addition of ammonia is considerably lower than normal (Part I, Fig. 12).
5. Normal cells have a fairly low respiration rate which is unaffected by the addition of ammonia (Part I, Fig. 6).
6. When glucose is added to these cells a marked increase of the respiration rate occurs. The rate reached is much the same as that of nitrogen-starved cells which are assimilating ammonia (Part I, Table I).
7. The addition of ammonium sulphate has little effect on the fermentation rate of nitrogen-starved cells (Part I, Fig. 3).
8. Normal cells assimilate ammonia slowly in darkness. The rate of assimilation is considerably increased when glucose is added or the cultures illuminated (Part I, Figs. 5 and 13).
9. Nitrogen-starved cells assimilate ammonia rapidly in darkness. The rate of assimilation is hardly affected by the addition of glucose or by illumination (Part I, Figs. 7, 13, and 14).

With these facts in mind, several possible explanations of this effect can be considered.

1. The possibility that the increased respiration rate which follows the addition of ammonia is due to the synthesis of respiratory enzymes can at once be rejected. The increase of respiration occurs as soon as the ammonium salt is added, yet little protein synthesis occurs during the first 20 minutes of ammonia assimilation (Figs. 1 and 2). Also the respiration rate returns to its original value when all the ammonia added has disappeared (Part I, Figs. 7 and 9). Clearly the high respiration rate is connected with the actual presence of ammonium ions.

2. It is possible that ammonium ions stimulate some enzyme systems involved in respiration. That such an effect can occur has been shown by several workers. Muntz (1947) showed that ammonium ions stimulate fermentation by yeast juice and Muntz and Hurwitz (1951) have studied the stimulatory effect of ammonium ions on several reactions of glycolysis. Whenever such effects have been found, however, it has been shown that potassium ions will also stimulate the enzyme system. Since in these experiments with *Chlorella* the concentration of potassium ions was high, such a stimulatory effect of ammonium ions would appear to be unlikely. Also, if such an effect occurs one must explain the low respiration rate of normal cells in the presence of ammonium ions. One might argue that normal cells contain little substrate for endogenous respiration and consequently no stimulatory effect of ammonium ions can be observed. While this might be true of cells used in the experiments described here, it cannot be true of cells used for previous work (Syrett, 1951). These were allowed to assimilate glucose in the presence of ammonium ions. Much of the glucose was assimilated to polysaccharide. When all the glucose had disappeared the respiration rate returned to the low endogenous rate; yet the cells now contained reserve carbohydrate in the presence of ammonium ions.

3. Another possibility is that a certain carbon compound accumulates in nitrogen-starved cells but not in normal cells, and that this is rapidly respired when ammonia is added. Although this idea is not so easy to reject as the previous two, it nevertheless appears to be unlikely. It is not easy to see how the addition of ammonia can suddenly lead to the oxidation of such a substance unless the activity of some enzyme system is stimulated; this is unlikely to happen for the reasons discussed above. Secondly, when ammonia is added to a small quantity of cells a high rate of respiration and rapid ammonia assimilation follow. These continue until the carbon reserve within the cell is exhausted (Part I, Fig. 8); the respiration rate then returns to a low level. When glucose is added the high respiration rate and assimilation continue until all the ammonia has gone. If the high rate of respiration is merely connected with the metabolism of a carbon compound peculiar to nitrogen-starved cells, it should cease when this compound has disappeared whether glucose is present or not.

4. Gregory and Sen (1937) have suggested that the rate of respiration of barley leaves is controlled by the rate of a protein cycle. It is thought that protein breakdown is continuous, the carbon residues formed being respired to carbon dioxide; at the same time more protein is synthesized from ammonia and other carbon residues which are produced from carbohydrate. Thus it is the rate of protein breakdown which controls the rate of carbon dioxide production.

If the addition of ammonia to nitrogen-starved cells of *Chlorella* greatly accelerated such a protein cycle, the greatly increased respiration might be explained. Such an explanation is unlikely, however. The highest rate of respiration occurs immediately after the ammonia is added and at a time when there is no net synthesis of protein (Figs. 1 and 2). It seems unlikely that both protein breakdown and protein synthesis increase by some four- or five-fold during this period, yet such an increase must be postulated if the high respiration rate is to be explained in this way. When ammonia assimilation has ceased the rate of respiration drops almost to its original value; yet the amino-acid concentration is now high (Figs. 1 and 2) and protein synthesis presumably active. Thus the high rate of respiration is associated with the presence of ammonium ions and may be connected with their assimilation (see also Part I, Fig. 5).

5. Since these various interpretations do not appear to explain the results a further possibility must be considered.

Figs. 1 and 2 and Table I show that amide, especially glutamine, is synthesized rapidly after the addition of ammonia. Chibnall (1939) has suggested that the synthesis of glutamine by *Lolium* leaves is accompanied by an increase of respiration rate. Willis (1951, and Yemm, 1949) has shown that glutamine synthesis in barley roots is accompanied by an increase in respiration and loss of carbohydrates.

Elliott (1951) has shown that glutamine synthesis is catalysed by enzymes which have been found in animals, bacteria, and higher plants. Adenosine-

triphosphate (A.T.P.) and adenosine-diphosphate (A.D.P.) are involved. The reaction is:

$$\text{glutamic acid} + \text{A.T.P.} + NH_3 \longrightarrow \text{glutamine} + \text{A.D.P.} + H_3PO_4.$$

These phosphorylated compounds are also involved in carbohydrate breakdown, and it has been suggested that the ratio of A.T.P. to A.D.P. may control the rate of this process (Johnson, 1941; see also Turner, 1951). Something like this may control the respiration rate of brain tissue (McIlwain, 1950). It is probable that glutamine synthesis is connected with respiration through such phosphorylated compounds (Yemm, 1949). It may well be that the addition of ammonia to nitrogen-starved *Chlorella* cells, which leads to the synthesis of amide, may result in the increased utilization of something like A.T.P. and hence an increased respiration rate.

Such an interpretation appears to explain the results obtained here but only further work can show whether it is correct.

Since this was written Dr. Yemm has proposed a similar explanation for the stimulation of respiration which follows the addition of ammonium salts to nitrogen-starved barley roots. (Meeting of the Society for Experimental Biology, Manchester, April 1952.)

Acknowledgements

The Warburg respirometer apparatus used in this work was purchased with a grant from the Dixon Fund of the University of London.

I should like to thank Professor W. H. Pearsall, Dr. G. E. Fogg, Dr. L. Fowden, and Mr. H. Tristram for their helpful criticism of the manuscript of this paper. I should also like to thank Miss L. Bishop for her technical assistance.

Literature Cited

Arnon, D. I., 1938: Microelements in Culture-solution Experiments with Higher Plants. Amer. J. Bot., xxv. 322.

Chibnall, A. C., 1939: Protein Metabolism in the Plant, p. 210. New Haven: Yale University Press.

Conway, E. J., 1947: Microdiffusion Analysis and Volumetric Error. London: Crosby Lockwood & Son, Ltd.

Cramer, M. L., and Myers, J., 1948: Nitrate Reduction and Assimilation in *Chlorella*. J. Gen. Physiol., xxxii. 93.

Dixon, M., 1943: Manometric Methods, 2nd ed., p. 49. Cambridge University Press.

Elliott, W. H., 1951: Studies in the Enzymic Synthesis of Glutamine. Biochem. J., xlix. 106.

Emerson, R., 1927: The Effect of Certain Respiratory Inhibitors on the Respiration of *Chlorella*. J. Gen. Physiol., x. 469.

Fisher, R. A., 1946: Statistical Methods for Research Workers, 10th ed., p. 128. Edinburgh: Oliver & Boyd.

Folin, O., and Denis, W., 1916: Nitrogen Determinations by Direct Nesslerization. J. Biol. Chem., xxvi. 473.

Frame, E. G., Russell, J. A., and Wilhelmi, A. E., 1943: The Colorimetric Estimation of Amino-nitrogen in Blood. Ibid., cxlix. 255.

Fujita, A., and Iwatake, D., 1931: Bestimmung des echten Blutzuckers ohne Hefe. Biochem. Z., ccxlii. 43.

Gregory, F. G., and Sen, P. K., 1937: Physiological Studies in Plant Nutrition VI. The Relation of Respiration Rate to the Carbohydrate and Nitrogen Metabolism of the Barley Leaf as determined by Nitrogen and Potassium Deficiency. Ann. Bot., n.s., i. 521.

Hagedorn, H. C., and Jensen, B. N., 1923: Zur Microbestimmung des Blutzuckers mittels Ferricyanide. Biochem. Z., cxxxv. 46.

Hamner, K. C., 1936: Effects of Nitrogen Supply on Rates of Photosynthesis and Respiration in Plants. Bot. Gaz., xcvii. 744.

Johnson, M. J., 1941: The Role of Aerobic Phosphorylation in the Pasteur Effect. Science, xciv. 200.

McIlwain, H., 1950: Brain Metabolism and Activity. Brit. Med. Bull., vi. 301.

McLean, D. J., and Fisher, K. C., 1947: The Relation between Oxygen Consumption and the Utilisation of Ammonia for Growth in *Serratia marcescens*. J. Bact., liv. 599.

—— 1949: The Extra Oxygen consumed during Growth of *Serratia marcescens* as a Function of the Carbon and Nitrogen Sources and of Temperature. Ibid., lviii. 417.

Moore, S., and Stein, W. H., 1948: Photometric Ninhydrin Method for Use in the Chromatography of Amino Acids. J. Biol. Chem., clxxvi. 367.

Muntz, J. A., 1947: The Role of Potassium and Ammonium Ions in Alcoholic Fermentation. Ibid., clxxi. 653.

—— and Hurwitz, J., 1951: Effect of Potassium and Ammonium Ions upon Glycolysis catalysed by an Extract of Rat Brain. Arch. Biochem., xxxii. 124.

Myers, J., 1949: The Pattern of Photosynthesis in *Chlorella*. Photosynthesis in Plants, p. 349. Iowa State College Press.

Robbie, W. A., 1946: The Quantitative Control of Cyanide in Manometric Experimentation. J. Cell. Comp. Physiol., xxvii. 181.

Roine, P., 1947: On the Formation of Primary Amino Acids in the Protein Synthesis in Yeast. Ann. Acad. Sci. Fennicae, Ser. A, II. Chem., No. 26.

Russell, J. A., 1944: Note on the Colorimetric Estimation of Amino-nitrogen. J. Biol. Chem., clvi. 467.

Said, H., and El-Shishiny, E. D. H., 1948: The Effect of Temperature on Respiration and Nitrogen Metabolism of Radish Root Slices. Bull. Faculty of Science, No. 27. Fouad I University.

Sankaran, R. A., 1936: The Respiration Changes induced in Phosphorus Deficient Leaves of Barley by feeding with Various Nutrients. Ph.D. Thesis, London University.

Steward, F. C., and Street, H. E., 1946: The Soluble Nitrogen Fractions of Potato Tubers: the Amides. Plant Physiol., xxi. 155.

Syrett, P. J., 1951: The Effect of Cyanide on the Respiration and the Oxidative Assimilation of Glucose by *Chlorella vulgaris*. Ann. Bot., n.s., xv. 473.

Turner, J. S., 1951: Respiration (The Pasteur Effect in Plants). Ann. Rev. Plant. Physiol., ii. 145.

Umbreit, W. W., Burris, R. H., and Stauffer, J. F., 1949: Manometric Techniques and Tissue Metabolism, p. 19. Minneapolis: Burgess Publishing Co.

Vickery, H. B., Pucker, G. W., Clark, H. E., Chibnall, A. C., and Westall, R. G., 1935: The Determination of Glutamine in the Presence of Asparagine. Biochem. J., xxix. 2710.

White, H. L., 1936: The Interaction of Factors in the Growth of *Lemna*. VIII. The Effect of Nitrogen on Growth and Multiplication. Ann. Bot., l. 403.

—— and Templeman, W. G., 1937: The Interaction of Factors in the Growth of *Lemna*. X. The Interaction of Nitrogen and Light Intensity in Relation to Respiration. Ann. Bot., n.s., i. 191.

Willis, A. J., 1951: Synthesis of Amino-acids in Young Roots of Barley. Biochem. J., xlix, p. xxvii.

Yemm, E. W., 1949: Glutamine in the Metabolism of Barley Plants. New Phytol., xlviii. 315.

Experimental and Analytical Studies of Pteridophytes

XIX. Investigations on *Marsilea*

2. *Induced Reversion to Juvenile Stages*

BY

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With Plate I and five Figures in the Text

Abstract

It is shown that in plants of *Marsilea* growing in aseptic culture reversion to juvenile leaves may be obtained by depriving the cultures of either sugar or mineral nutrients. Reduction to a protostele and other anatomical observations are also described.

Juvenile leaves were produced by excised apices of mature plants, and by lateral branches developing on feeble decapitated plants. The problem of heteroblastic development is discussed in relation to these and other observations.

Introduction

IT has already been shown (Allsopp, 1952) that the characteristic heteroblastic development of sporelings of *Marsilea* is greatly influenced by culture in media containing suitable amounts of various growth-regulating

[Annals of Botany, N.S. Vol. XVII, No. 65, January, 1953.]

substances. In view of the low concentrations required, and since the resulting deviations from normal development were always accompanied by considerable changes in the root system, it was concluded that the effects on leaf morphology were a secondary result of the induced changes in root development. The hypothesis was advanced that the abnormalities in leaf morphology were produced by impaired nutrition, either as a consequence of the lack of an adequate absorbing system or in some cases as a result of diversion of materials from developing leaf primordia into the supernumerary induced roots.

The evidence obtained from this earlier work, although of an indirect character, thus indicated that the morphology of leaves may be modified by the supply of nutrients and that the heteroblastic development of many plants may be nutritional in origin. Most previous workers on this subject have attempted to modify the nutritional status of the plant by changing such environmental conditions as light intensity, temperature of the soil, &c. The methods of sterile culture now admit a more direct attack.

In the present studies two approaches have been followed. In one group of experiments the development of the sporeling was influenced by changing the sugar concentration of the culture medium, with other conditions constant. This aspect of the work will be dealt with in a later contribution. In another group the nutrition of more mature plants was reduced under controlled conditions. This work is described in the following account.

Materials and Methods

The work was carried out on *Marsilea Drummondii* A.Br.

Sterile cultures were obtained by the method described previously (Allsopp, 1952). Unless otherwise indicated, the basic culture medium was the same as in the earlier work.

Excision of apices and other surgical treatments were carried out after transferring the cultures to sterile Petri dishes. The prepared materials were then transferred to fresh culture tubes.

I. Starvation Experiments

The hypothesis was advanced by Goebel (1898) that the juvenile leaves of plants showing heteroblastic development are arrested structures whose growth is restricted by the limited nutritional powers of the small young plant. On this view it might be expected that starvation of mature plants would result in a reversion to juvenile leaves. This belief was tested by the following experiments.

(*a*) *With complete plants.* Plants which had been grown for 3 months on the basic medium containing various concentrations of glucose were transferred to the same basic medium but with entire omission of sugar or other organic carbon source. The following plants were used: 2 from 1 per cent. glucose, which had produced 25 and 22 leaves respectively; 2 from 2 per cent. glucose

with 28 and 21 leaves; 2 from 3 per cent. glucose with 25 and 20 leaves; and 1 from 5 per cent. glucose with 18 leaves. At the time of transfer all plants were growing well and producing leaves each with a quadrifid lamina.

In the earlier paper (Allsopp, 1952) it was shown that the light-intensity of the culture room was insufficient to support normal sporeling growth unless an organic carbon source was supplied. In the present experiment, however, the plants continued to grow and produce new quadrifid leaves. The leaves showed a gradual reduction in size, particularly of the lamina, and where internodes were originally conspicuous they now suffered a progressive shortening. The rate of leaf formation steadily diminished, but even after 5 months in all but one plant, from 3 per cent. glucose, the leaves were still quadrifid. The plant from 3 per cent. glucose had formed 14 quadrifid leaves after transfer, but leaf 15 and subsequent leaves were now bifid.

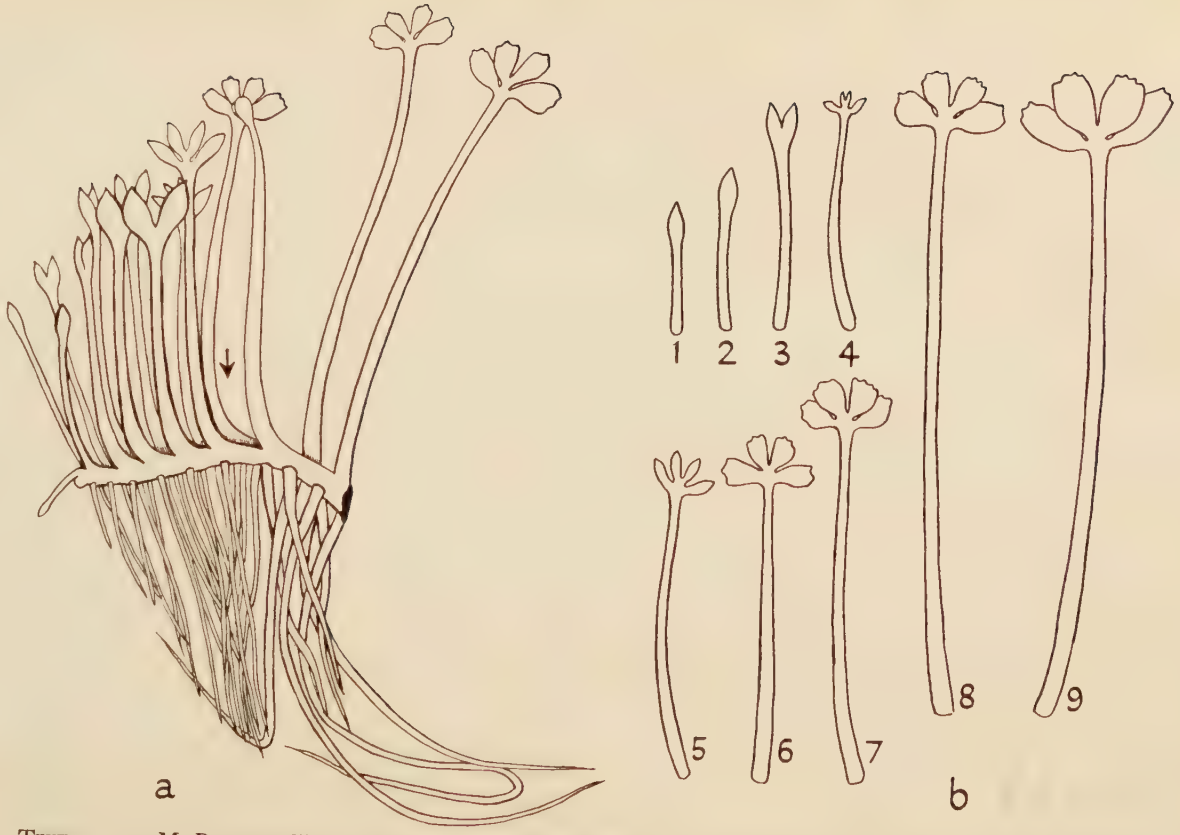
Pl. I, Fig. 1, illustrates the plant from 5 per cent. glucose 3 weeks after transfer to the inorganic medium. The abrupt change from the land-leaf form to the water-leaf form is very striking, but discussion of this feature will be postponed till a later contribution.

It was evident from the above experiment that intact plants of *Marsilea* can continue growth for a long period, even when deprived of an adequate external source of carbohydrate, by drawing on the reserve materials already present in the plant body. In every plant the older parts shrivelled and became yellow as their materials were transferred to the actively growing regions.

(*b*) *With terminal lengths of rhizome.* The experiment described in section (*a*) showed that complete plants would be expected to yield the juvenile type of leaf only after a long exposure to conditions of carbohydrate starvation. In the present series of experiments the available reserve materials were reduced by transferring only short terminal lengths of the rhizome to starvation conditions. The predicted formation of juvenile leaves was then realized (Text-fig. 1 (*a*)).

From the figure it will be seen that after transfer to conditions of carbohydrate starvation a gradual attenuation of all parts sets in. The roots become slender and shorter and the diameter and length of the internodes of the rhizome is reduced. But the leaves show the most pronounced reaction. The first 3 leaves produced in the new medium are all quadrifid but are successively smaller, then follow 6 bifid and finally 3 simple undivided leaves.

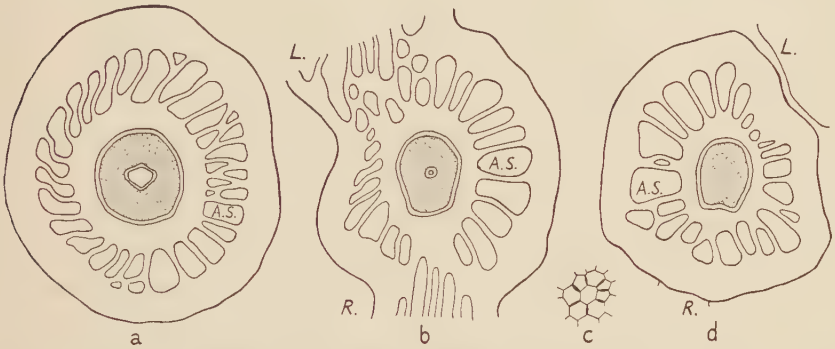
Shorter lengths of rhizome yielded essentially similar results, but as might be expected the reduction proceeded more rapidly. Thus, two lengths of rhizome when transferred to starvation conditions had each one expanded leaf and one coiled primordium. A final examination 10 months after transfer showed that one plant had produced 3 additional quadrifid, 1 bifid, and 4 simple leaves. The other plant, Text-fig. 1 (*b*), had formed in succession 4 quadrifid, 1 bifid, and 2 simple leaves. Both plants showed also a progressive attenuation of all parts, and as in the case of the plant described in the previous paragraph, the rate of leaf formation had gradually diminished to a very low level.



TEXT-FIG. 1. *M. Drummondii.* (*a*) Growth produced by a short terminal length of rhizome after 9 months in basic medium with sugar omitted. Parts to left of arrow were formed after transfer. (×4.) (*b*) Successive leaves (numbered from apex) produced by a shorter length of rhizome under similar conditions. Leaves 8 and 9 were present before transfer. (×4.)

It is clear from these results that after the reserves of the plant are exhausted, lack of carbohydrate induces a gradual reversion to the juvenile leaf form. There is a complete reversal of the normal development, and indeed, when taken from the apex backwards, the leaf succession illustrated in Text-figs. 1 (*a*) and (*b*) closely resembles that observed in normal sporeling development.

(*c*) *Anatomical observations.* The plants described in section (*b*) above, and illustrated in Text-figs. 1 (*a*) and (*b*), were examined by serial microtome sections. In both plants the gradual changes in external morphology were



Text-fig. 2. *M. Drummondii.* Successive transverse sections from plant illustrated in Text-fig. 1 (*a*). (*a*) At internode between leaves 14 and 15 (numbered from apex). (*b*) At insertion of leaf 8. (*d*) Near insertion of leaf 5. (All ×80. A.S., Large cortical air spaces; L, insertion of leaf; R, insertion of root. Stele dotted. Endodermis and medulla clear.) (*c*) Enlarged view (×300) of inner endodermis and single medullary cell of (*b*).

accompanied by corresponding anatomical changes. Text-figs. 2 (*a*)–(*d*) and Pl. I, Figs. 2–4, illustrate some of the anatomical features of the plant shown in Text-fig. 1 (*a*).

As shown in the Figures a reduction in size of the stele follows attenuation of the rhizome. A section taken in the region of the rhizome already present before transfer to starvation conditions reveals a typical amphiphloic solenostele with an inner endodermis surrounding a small triangular pith. The pith persists for a time after transfer but is gradually reduced in passing towards the apex. Thus in the section shown in Pl. I, Fig. 2, at the level of the thirteenth leaf from the apex (L. 13), there are 16 pith cells. Between L. 10 and 9 the pith is reduced to 7–9 cells, between L. 9 and 8 to 3–4 cells, and finally between L. 8 and 7 to 1 cell. (Text-figs. 2 (*b*) and (*c*) and Pl. I, Fig. 4 are from sections at this level.) In this region the endodermis, originally of many cells, is reduced to 6 cells surrounding the single cell of the pith (Text-fig. 2 (*c*)). The endodermis is lost entirely between L. 7 and 6, and we thus pass to the condition of a medullated protostele. During this transition the xylem elements, strongly thickened and lignified in the region of the rhizome, developed before transfer, become thinner-walled and less strongly lignified. In the protostelic region, L. 6–apex, it is not easy to distinguish the xylem elements

in a transverse section, but it is clear that the medullation is reduced and central tracheides are found between L. 3 and the apical region.

The reduced dimensions of the rhizome are a result of a decrease in cell number rather than in cell size. This is at once evident from a consideration of Pl. I, Figs. 2 and 4, and is even more strikingly shown when sections nearer the apex are taken into account. The number of cortical air channels is also greatly reduced in passing towards the apex. This feature is shown to some extent by a comparison of Text-fig. 2 (*a*) and (*d*) but becomes more pronounced in sections nearer the apex.

The behaviour of the lateral growing points is also of considerable interest. In *Marsilea* these are normally situated laterally on the rhizome, below and slightly in advance of each leaf-base. The apical cell of the lateral meristem is differentiated at an early stage from the same dorso-lateral segment of the apical cell of the rhizome as that which gives rise to the neighbouring leaf (Schneider, 1913). Development of the lateral meristem continues until a small bud is formed, when further growth is usually arrested for a period unless the inhibiting influence of the rhizome apex is removed, for example, by excision of the growing-point.

In the two starved plants investigated anatomically there is a progressive reduction in the size of the lateral bud in passing towards the attenuated region of the rhizome. The section shown in Pl. I, Fig. 2, passes through a lateral bud at the level of L. 13 of the main rhizome. The leaf primordium of the lateral, even at this early stage, shows the initiation of the two proximal lobes of the lamina, while an indentation at the tip of the primordium marks the point of separation of the two distal lobes. Pl. I, Fig. 3, illustrates the condition of the lateral associated with L. 12, the first leaf to develop under starvation conditions. The whole lateral is already considerably reduced, and its leaf primordium is a simple rounded structure with no indication of the lobing so clearly seen in the previous section. The reduction of the laterals continues at the two following nodes, where the leaf primordium of each lateral is represented only by a small projection of the growing-point. At L. 9 the lateral meristem is further reduced and a leaf primordium can be detected no longer. In passing towards the apex the lateral meristems become successively smaller. Pl. I, Fig. 4, illustrates the condition at L. 8. Finally L. 2 and L. 1 show no associated lateral meristem. The other plant investigated anatomically shows a similar reduction of the lateral meristems; but as with the other changes described above, the sequence is more rapid, as might be expected from the smaller length of rhizome originally transferred.

Another interesting feature is shown by the vascular supply of the lateral meristems. A small vascular strand passes into the rhizome from each lateral meristem and joins with the main stele slightly in advance of the corresponding leaf trace. In both of the investigated plants, at nodes where lateral meristems were lacking, there was never the slightest trace of the vascular strands which normally lead out to these meristems.

II. Experiments with Reduced Mineral Nutrition

In the experiments described above reversion to juvenile leaves was induced by omitting sugar from the culture medium. The present series was commenced with the object of determining whether leaf morphology can be influenced by changes in the concentration of mineral constituents of the medium.

Parallel sets of 8 cultures, all from the same sporocarp, were grown on a normal sugar concentration of 2 per cent. glucose but with the Knop's solution at $\frac{1}{5}$, 1, 5, and 10 times the normal concentration respectively. Other details of this experiment will be given in a later contribution.

As would be expected, cultures inoculated on 2 per cent. glucose and distilled water only, died very quickly after producing only a few simple leaves. In the 10× Knop's solution leaf development was abnormal, but in the other three concentrations the rate of leaf formation and the appearance of divisions in the lamina followed a very similar course during the first few months. A typical culture from the normal Knop's solution after 6 months of growth is illustrated in Pl. I, Fig. 5. Apical growth and formation of quadrifid leaves was still continuing actively. The cultures on the medium containing $\frac{1}{5}$ Knop's however, all showed a sudden reduction in growth rate, although the leaves produced during the early phase of growth were rather larger than in the normal medium. This reduction in growth rate almost certainly coincided with exhaustion of one or more of the mineral nutrients; tests revealed that there was still an ample supply of sugar in the medium. Following the reduction in growth rate, death of the tissues, as indicated by a shrivelling and browning of the affected parts, quickly spread from the base towards the apex of the plant.

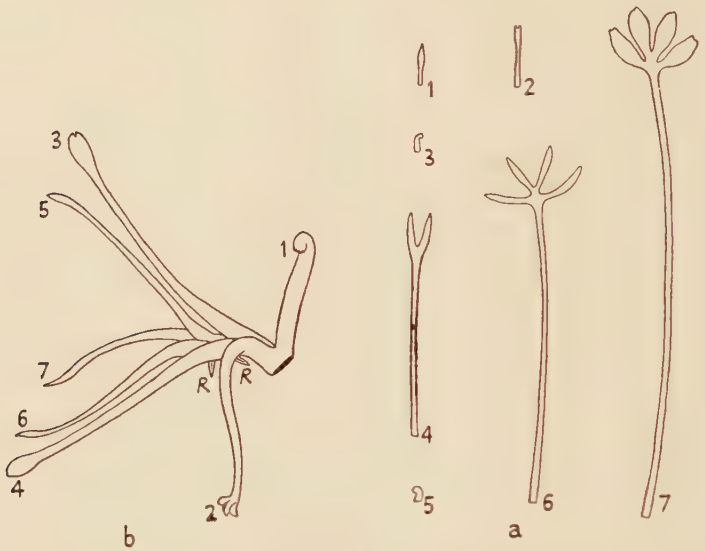
In some cultures cessation of growth was abrupt. In others there was a more gradual falling away accompanied by the formation of successively simpler leaves. Such a plant is shown in Pl. I, Fig. 6, where a bifid leaf is distinctly visible near the apex. The final seven leaves from this plant are illustrated in Text-fig. 3 (*a*). The seventh leaf from the apex (L. 7) is a normal quadrifid water-leaf similar to those already produced by this plant, while the later leaves show a progressive reduction. Thus in this small plant an increase in leaf complexity from an undivided through a bifid to a quadrifid leaf was followed by a corresponding reversal on exhaustion of one or more of the mineral nutrients.

III. Growth of Excised Apices

In section 1 (*b*) it was shown that when terminal lengths of the rhizome are transferred to a medium without sugar or other organic carbon source, growth is continued for a time and the gradual exhaustion of the reserve materials is accompanied by a reversion of the leaves to more juvenile stages. In the present section the development of isolated rhizome apices is described.

The apex of *Marsilea* has been described by several workers, notably by

Schneider (1913), and the existing literature has been reviewed recently by Tournay (1951). It is known from these accounts, which have been confirmed in a general way by the present author, that the segmentation at the apex of *Marsilea* is very regular, and that leaves, roots, and lateral meristems are initiated close to the apical cell, one root and one lateral meristem in association with each leaf. Thus even the terminal millimetre of the rhizome carries minute leaf and root primordia.



TEXT-FIG. 3. *M. Drummondii.* (*a*) Successive leaves (numbered from apex) from plant grown on 2 per cent. glucose medium with $\frac{1}{5}$ of the normal supply of mineral nutrients (×3). (*b*) Growth produced by excised apex after 10 months in basic medium with sugar omitted. Leaves numbered in order of appearance. Plant had previously been grown for 4 months on medium containing 3 per cent. glucose (×6).

In the present series of experiments the terminal millimetre of the stem was excised under aseptic conditions. The growing-points used carried no external roots and only one externally visible unexpanded leaf primordium. In one treatment the apices were transferred to inorganic media only; in the other to 2 per cent. glucose media. The effects of wounding were apparently slight and growth was resumed at once.

(*a*) *In inorganic media.* Apices (1 mm.) were removed from plants which had been cultured for 3 months on media containing various concentrations of glucose. The following plants were used: 2 from 1 per cent. glucose; 2 from 2 per cent.; 2 from 3 per cent.; and 2 from 5 per cent. After excision the apices were transferred to a normal mineral medium lacking sugar or other organic carbon source.

In each case development was similar and exceedingly slow. A typical culture, 10 months after transfer from 3 per cent. glucose is illustrated in Text-fig. 3 (*b*). In this plant the first two leaves expanded after transfer still

show vestiges of a quadrifid lamina. It is believed that the quadrifid pattern had already been established in the primordia, which failed to expand normally on account of inadequate nutrition. The third leaf is better developed, but the lamina is simple and spatulate with only a slight terminal notch. Leaf 4 is also spatulate, but later leaves, L. 5–7, are successively smaller and subulate with no differentiation of a distinct lamina. Root development is very slight, while the axis becomes very attenuated and internode extension is suppressed. The other apices behaved very similarly with only small differences in the number of leaves produced.

The growth of isolated apices on transfer to a purely inorganic medium thus closely resembles that of an embryo developing on the same medium (Allsopp, 1952, Text-fig. 2). That the formation of juvenile leaves by isolated apices when grown under such conditions is a consequence of carbohydrate starvation is shown by the results described in the following section.

(*b*) *In sugar solutions.* A number of excised apices, approximately 1 mm. in length, were transferred to the standard basic medium containing 2 per cent. glucose.

In all cases fairly rapid development commenced at once. The first few leaves appearing after transfer were invariably quadrifid, but feebly developed, especially in the lamina. Their appearance suggested that they had already been initiated at the time of transfer, but that the rate of entry of nutrients into the as yet rootless excised apices was insufficient to admit of their normal development.

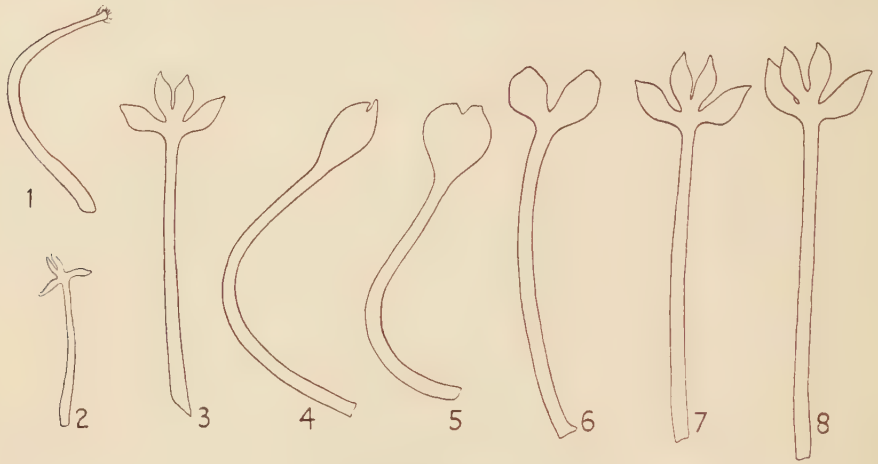
In some apices the first few abnormal leaves were followed by successively larger quadrifid leaves until the normal development was again attained. In the majority of apices simpler leaves appeared before a return to the usual quadrifid condition. Thus in Text-fig. 4 (*a*) the fourth leaf produced after transfer (L. 4) has a simple spatulate lamina with only a slight terminal notch. L. 5 has a broader lamina with larger terminal depression, L. 6 is bifid, while L. 7 and 8 have each a quadrifid lamina but are smaller than the leaves produced before excision.

In Text-fig. 4 (*b*) a similar sequence is followed. In this culture L. 7 reverted to the bifid condition, but L. 8 and all subsequent leaves were quadrifid.

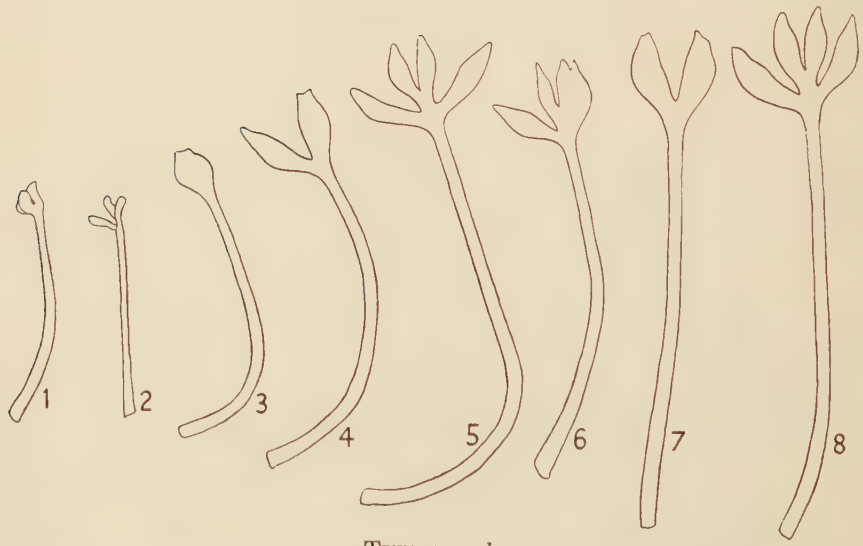
The simple and bifid leaves of the two figured, as well as of other cultures, were healthy organs and showed no indication of arrested growth during development from the primordium. It would seem rather that in the excised apex the expansion of the leaf primordia already present draws heavily on the available nutrients. The cut surface of the apex is evidently of little value in the supply of nutrients and the leaf primordia produced after the expansion of the pre-existing primordia arise under starvation conditions and are correspondingly reduced. With the subsequent appearance of new roots the nutrition of the apex is improved and there is a gradual increase in leaf complexity.

The plants developed from isolated apices are thus very similar to sporelings, and all organs, including stem and roots, are considerably reduced. This

point is emphasized by a comparison of Pl. I, Fig. 7, which illustrates the culture whose leaves are represented in Text-fig. 4 (*a*) with Pl. I, Fig. 5, which is a normally developed plant similar to that from which the apex was excised.



TEXT-FIG. 4 *a*



TEXT-FIG. 4 *b*

TEXT-FIG. 4. *M. Drummondii*. Successive leaves, in order of appearance, from excised apices grown in 2 per cent. glucose media. (*a*) From plant previously grown in 1 per cent. glucose medium. (*b*) Previously in 3 per cent. glucose (× 5).

IV. Observations on Lateral Branches

The position of the lateral meristems of *Marsilea* has been described already in section 1 (*c*). After producing small buds the meristems usually remain

dormant under conditions of culture unless the apex of the main rhizome is removed. Activity then commences in nearly all the laterals, but one or a small number, usually near the apex, gain the ascendancy and the others are suppressed. When plants are cut into segments, however, each containing one node with its dormant lateral, a new plant is usually produced from the lateral bud of each segment.

Experiments were carried out to determine whether morphological position or nutritional history would influence the type of leaf produced by the laterals.

(*a*) *On decapitated plants.* Plants which had been grown for 4 months on culture solutions containing 1, 2, 3, and 5 per cent. glucose respectively were decapitated and all transferred to media containing 2 per cent. glucose.

Of the four plants grown on 1 per cent. glucose, which were feebly developed as compared with those from the 2 and 3 per cent. concentrations, two produced laterals commencing with simple spatulate leaves. Later these laterals gave rise to bifid leaves and finally to normal quadrifid leaves. The other two plants had laterals commencing with bifid leaves, later followed by quadrifid leaves.

Of the three plants from 2 per cent. glucose, two developed laterals commencing with bifid leaves, while the remaining plant, which was more robust, produced laterals with quadrifid leaves from the outset.

Only two plants were available from 3 per cent. glucose. Both had laterals with quadrifid leaves from the outset.

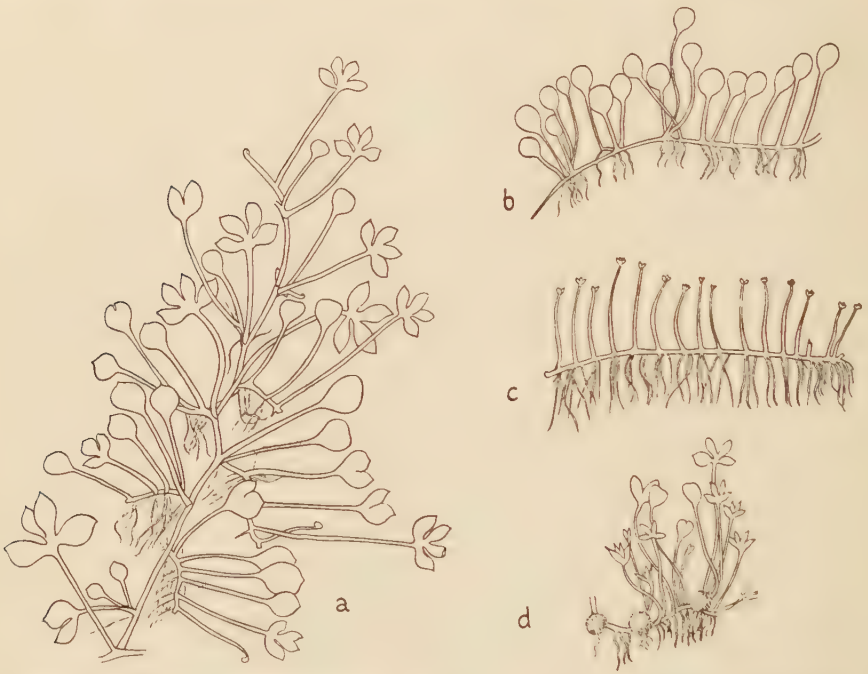
Plants grown on 5 per cent. glucose are usually somewhat stunted. It was therefore not surprising to find that of the five plants used, two produced laterals commencing with bifid leaves, while the other three plants had laterals with quadrifid leaves from the outset.

Although the above experiment is admittedly of a preliminary character, it demonstrated quite clearly that laterals developing from feeble plants produce leaves of a more juvenile type than do laterals from robustly developed plants. The leaf sequences of feeble laterals are similar to those of normal sporeling development; also to those illustrated in Text-figs. 4 (*a*) and (*b*) for isolated apices. The laterals obtained in nature by Glück (1911) and illustrated in Text-fig. 5 (*a*) are also very similar.

(*b*) *On decapitated plants under starvation conditions.* The experiment described in this section was a duplicate of that recorded in section (*a*), but with omission of sugar from the medium.

As might be expected from the results obtained in section I (*a*), the reserve materials of the rhizome were sufficient to support the long-continued growth of the laterals. The extent of division of leaf-blades of the laterals was at first entirely unaffected by the starvation conditions, but was related to the previous nutrition in exactly the same way as described for the plants of section (*a*). With further growth, however, there was a gradual shortening of the internodes and decrease in size of the leaves, followed in some cases by a reversion to a more juvenile leaf type.

(*c*) *On isolated rhizome segments.* In this section observations were made on the growth of isolated segments of the rhizome, each segment consisting of a node and portions of the adjoining internodes. The node carried one leaf, a few roots, and the lateral bud. The first few nodes at the base of the plant were treated as one unit. The segments were obtained from the following



Text-fig. 5. (*a*), (*b*) *Marsilea hirsuta* f. *submersa*. (*a*) After 2 months at a water depth of 70 cm. (× ⅔). (*b*) After 1 year at a water depth of 70 cm. (× ⅔). (*c*) *M. pubescens* f. *submersa*. After 1 year at a water depth of 70 cm. (× ⅔). (*d*) *M. paradoxa*. Plant from natural habitat with juvenile leaves and pill-shaped sporocarps. Figs. (*a*)–(*c*) from Glück (1911); (*d*) from Diels (1906).

materials: one plant from 1 per cent., one from 2 per cent., and three from 3 per cent. glucose media. All were transferred to 2 per cent. glucose medium.

All the segments produced a lateral branch. In every case segments 1–8, numbered from the apex, gave rise to laterals commencing with quadrifid leaves. But segments nearer the base produced laterals commencing with a simpler type of leaf regardless of the previous sugar concentration. Thus segment 9 of a 3 per cent. glucose plant produced three simple leaves followed by two bifid before arriving at the quadrifid leaf stage, and the corresponding segment of another plant from 3 per cent. glucose produced four simple leaves and six bifid before the quadrifid condition was attained.

The results from the single plant from 1 per cent. glucose are of interest. The lateral branches were at first less well developed than those produced by segments from plants previously grown on higher sugar concentrations. In

some segments the less vigorous development was reflected in a reversion to a more juvenile type of leaf. Thus in segments 2 and 7 the first leaf of each lateral was quadrifid, and was succeeded by two and one bifid leaves respectively, followed in turn by quadrifid leaves again. These results may well indicate that the reserves of the rhizome segments from the lower sugar concentration are inadequate for the continued production of quadrifid leaves. The quadrifid leaf condition is regained when the nutrition of the lateral is improved by the development of its own root system.

This explanation is supported by the behaviour of segments, obtained from a plant previously grown on 3 per cent. glucose, in which the leaf and roots were excised from each segment before transfer. The results were similar to those obtained with the plant from 1 per cent. glucose. The first leaf produced by the lateral from each segment was quadrifid but was followed by a bifid leaf before a stable quadrifid condition was attained. This behaviour is similar to that described for isolated apices. In both cases a persistent quadrifid condition is attained only when the active meristem is supplied by an adequate root system.

Discussion

The results obtained in the present study are of interest in relation to the general problem of heteroblastic development; the term proposed by Goebel (1898) for that type of plant development in which the juvenile stages, especially of the leaves, show relatively considerable differences from the adult form. Goebel and his associates carried out many investigations on this subject, which are summarized in the third edition of the *Organographie der Pflanzen* (Goebel, 1928). Subsequent work has been considered critically by Ashby (1948).

Goebel believed that most cases of heteroblastic development are related to the nutrition of the plant. All leaves of the plant were said to show the same pattern of development, but only in the mature leaves is this continued through the complete sequence of stages characteristic of the species. The juvenile leaves are arrested formations whose development is interrupted at one or other of the intermediate stages. Goebel found that in many plants the higher type of leaf is reached more quickly the stronger the germ plant. Furthermore, by placing plants under unfavourable conditions he was able to induce a reversion to the juvenile leaf form. Detailed accounts of many experiments of this type are given in Goebel (1908).

The extensive evidence amassed by Goebel and other workers provides almost overwhelming support for the hypothesis that heteroblastic development is related to the nutritional capacity of the developing plant. But the precise character of the nutritional effect has never been elucidated. In the present work additional information has been provided by taking advantage of the methods of sterile culture in applying various starvation treatments under otherwise constant conditions.

As reported in the experimental part of this paper, reversion to juvenile

leaves was obtained in *Marsilea* when complete plants were deprived of carbohydrate supplies. On account of the reserve materials already present in the plant, this was a slow process, but could be hastened by removing the basal parts of the plants leaving only one or more terminal segments. It was a striking feature of this reversion that the process provided a complete reversal of the normal heteroblastic development.

A similar reversion to juvenile leaves was observed by Glück (1911) when plants of *Marsilea hirsuta*, previously growing on land, were sunk in water to a depth of 70 cm. According to Glück, most species of *Marsilea* react by producing floating leaves, but *M. hirsuta* formed the characteristic water form illustrated in Text-figs. 5 (*a*) and (*b*). The appearance of juvenile leaves in *M. hirsuta* when grown under such conditions was considered by Glück to be restricted to this species and indeed of value in its systematic determination. This view is refuted by the present work and by the earlier observations of Woltereck (1928).

Glück obtained a different type of water form in *M. pubescens*. As illustrated in Text-fig. 5 (*c*) the submerged leaves were composed mainly of a fairly thick and subulate petiole which tapered above and bore a strongly reduced quadrifid, or simpler, lamina rarely 1 mm. in length. Similar water forms were found in *M. strigosa* and *M. diffusa*. These water forms are similar to the growth produced in the present work by starving plants of *Marsilea* before their reversion to a more definitely juvenile condition.

It has been emphasized by Goebel (1908, 1928) and other workers that the transformations obtained by submerging amphibious plants are not direct adaptations to the aqueous medium as such, but arise as a result of changes in metabolism which may also be induced in a variety of other ways. As factors that might be involved when plants are submerged, Glück (1911) listed water pressure, strongly diminished aeration, weakened light, and reduced temperature. He also considered that the reserve materials of the plant are important.

In the present investigation the production of juvenile leaves by changes in the carbohydrate supply only, indicates that the formation of the more juvenile 'water-leaf' type when amphibious plants are submerged may be referred to a condition of carbohydrate deficiency due almost entirely to the great reduction of light-intensity which is found even in fairly shallow water.

Woltereck (1928) carried out many investigations on the production of juvenile leaves in *Marsilea*. She obtained the interesting result that the mature leaf type could arise even in submerged plants when sufficient artificial lighting was supplied. But at an increased water temperature of 30–35° C. only juvenile leaf types were developed. She rightly considered that at the higher temperature there is so rapid a utilization of assimilates that the concentration remains inadequate for the formation of the more mature leaf type. Juvenile leaf types could also be maintained on land by growing the plants in a damp atmosphere with reduced light intensity. The nature of the

substratum was important in that formation of juvenile leaves was favoured by soils in which good rooting occurred. Reversion to juvenile leaves was achieved most easily by removing existing leaves of the mature type and raising the soil temperature to 30° C.

Woltereck considered that her results with *Marsilea*, and her analogous results with other amphibious plants, confirmed the view of Goebel that excess of assimilates leads to the formation of the mature leaf type, while excess of mineral nutrients causes inhibition of leaf development and reversion to the juvenile leaf form. The present writer, however, prefers an alternative explanation. In Woltereck's experiments a warm soil by increasing the respiration of the roots and probably also by promoting a more active root development would increase the deficiency of carbohydrates produced by the removal of leaves and the cutting down of the light. The conditions of carbohydrates starvation would then account for the reversion to juvenile leaves without the necessity of involving an increased supply of mineral nutrients.

The effects of reduced mineral nutrition reported in the experimental part of the present paper are relevant in this connexion. As in the experiments dealing with carbohydrate deficiency, juvenile leaves were obtained also in cultures provided with reduced supplies of mineral nutrients. In the light of these results the hypothesis is advanced that it is not merely carbohydrate deficiency that causes reversion to the juvenile leaf forms. Rather might reversion be referred to deficiency of any nutrient essential for protein synthesis, with consequent restriction of protein materials available to the leaf primordia at the growing-point.

Observations on reversion to juvenile leaves in pteridophytes have also been made by other workers. Thus Lang (1924) found that ill-nourished plants of *Osmunda regalis* may continue for a long period to bear leaves of the juvenile type, and that a return may also be made from a more advanced type of foliage to the juvenile leaves. Wardlaw (1945) obtained juvenile leaves from plantlings of *Onoclea sensibilis* which had been kept during the winter months in an incubator at 22–25° C., with ordinary daylight-illumination and a high relative humidity. In an investigation with *Dryopteris* apices (Wardlaw, 1949) young leaf primordia were isolated by four vertical incisions. These primordia showed very limited growth and developed into small awl-shaped parenchymatous structures. Reversion to juvenile leaves was also achieved in defoliation experiments in which shoots of *Osmunda regalis* were allowed to grow on after previous defoliation down to the smallest primordia (Wardlaw, 1946). Goebel (1908) described experiments in which the excised apices of *Ceratopteris* plants produced a more juvenile type of leaf on continued growth. These results are paralleled by the work with excised apices of *Marsilea*, which was considered in detail in section 3 of the present paper. In all the above examples the appearance of juvenile leaves was preceded by some disturbance of the normal nutritional relationships.

Goebel's hypothesis in its original form, however, will not account for all the described phenomena. Thus sporelings of *Marsilea* show a normal

heteroblastic development even when grown in solutions containing abundant supplies of carbohydrate (Allsopp, 1952). But observations of this character are readily explained by Troll's extension of Goebel's hypothesis.

Troll (1939, p. 1389) supported Goebel's view that heteroblastic development is determined largely by nutritional conditions, but emphasized that the transition from juvenile to adult leaves is related to the increase in size of the shoot axis. As the axis expands so does the growing-point. With increase in size of the growing-point there is a parallel increase in the size of the leaf primordia, which enables their subsequent development to continue for a longer period, thus leading to the attainment of the mature leaf form. Similarly any weakening of the axis, with concomitant reduction in the size of the growing-point, will be reflected in the reversion to juvenile leaf types. Examples of such reversion have been given above, and many further instances are described by Troll, pp. 1403–10.

It has been shown by Wardlaw (1948) that in leptosporangiate ferns the shoot apex undergoes a considerable enlargement during the development of the individual plant. He has recently concluded (Wardlaw, 1952) that 'whereas the primary morphogenetic activities of the apical region are closely comparable in large and small apices, very pronounced differences in leaf shape and size and in stelar morphology may be induced according to the nutritional status of the subapical region'. Thus the views of Wardlaw, based largely on extensive and detailed studies of apical phenomena, are in agreement with those advanced by Troll in explanation of the increase in leaf complexity occurring during normal heteroblastic development.

It is well known from the early and subsequent literature (summarized by Wardlaw, 1947) that in the normal ontogeny of a fern sporeling there is a gradual increase in stelar complexity. In *Marsilea*, as in most ferns, the germling commences with a protostele, but development of the vascular system does not proceed beyond an amphiphloic solenostele (Mahabale and Gorji, 1948; Allsopp, unpublished observations). In the present work the attenuated plants obtained by reduction in the carbohydrate supplies showed a gradual reversion from a solenostele to a protostele. A similar reversal of the normal course of vascular ontogeny has been described by Wardlaw for attenuated shoots of *Onoclea sensibilis*, *Osmunda regalis*, *Todea barbara*, and *Angiopteris evecta* in earlier papers of the present series of studies (summarized in Wardlaw, 1947). These changes in anatomy are associated with a reduction in size of the apical growing-point, but little is known of the precise factors involved.

Acceptance of the importance of the size of the apical growing-point eliminates certain difficulties which have been encountered in work dealing with lateral branches. Thus Lundegårdh (1915) considered that the quantity of available nutrients could not account for his observations, on *Ipomaea learii*, that the tendency of lateral buds of the first order to form mature leaves increases with their distance from the base of the main axis. A similar tendency has been found in *Marsilea* in the present work. But in the view of the writer

this tendency is adequately accounted for by the increase in size of the lateral meristems with increasing distance from the base of the plant.

In his work on *Ipomaea caerulea* Ashby (1950) found that the leaf shape on laterals is consistent with the hypothesis 'that leaves which differentiate their blades at the same time have the same shape, whether they are situated on the terminal bud or on lateral buds'. But although Ashby's data are not in conflict with this view, the hypothesis is not of general significance as there are many examples of the production of juvenile leaves on laterals in the axils of leaves of a more mature leaf type. Such an example is illustrated for *Marsilea* in Text-fig. 5 (*a*). The present writer would take the view that since the lateral growing-points are almost invariably smaller at their inception than the growing-point of the main axis, it is not surprising that we frequently observe juvenile leaves at the beginning of lateral branches.

A somewhat different approach has been adopted by the Russian worker Krenke (1933–5, 1940). According to Ashby (1948, 1950) Krenke in his 'theory of cyclic ageing and rejuvenescence' in plants considers that the rate of heteroblastic development may be used to measure the 'physiological age' of the plant and that various properties of the plant, e.g. earliness of flowering of cotton varieties, can be diagnosed from the shape of the leaves. 'An integral part of Krenke's hypothesis is that the first few leaves on lateral shoots are "physiologically younger" than leaves at the nodes from which they arise.'

The views of Krenke, although possibly of value in emphasizing the economic importance of observations on heteroblastic development, would appear to add little to the basic knowledge of this subject. Although the appearance of reproductive organs is often associated with a particular leaf shape, the two phenomena are a result of independent processes as clearly shown by the early work of Diels (1906). His example of the production of sporocarps by a plant of *Marsilea paradoxa* while still at a juvenile leaf stage is illustrated in Text-fig. 5 (*d*).

The present writer would take the view that in most cases of heteroblastic development it is unnecessary to seek explanations involving special growth-substances and mysterious processes of 'physiological ageing'. Changes in the size of the growing apex, as influenced by the changing nutritional status, would appear to supply an adequate explanation. This hypothesis, of course, is restricted to changes within the individual plant. Apart from certain genetical aspects, there is still virtually no information as to the factors responsible for the very great range of form in the leaves of different species.

Summary

When plants of *Marsilea* growing under aseptic conditions were transferred to conditions of carbohydrate starvation, reversion to juvenile leaves was obtained only after several months of exposure. Short terminal lengths of rhizome produced juvenile leaves more readily. The successively simpler leaves reversed the sequence of normal heteroblastic development. Anatomical

examination of the plants showed, along with other features, that the normal amphiphloic solenostele was gradually reduced to a protostele.

Plants grown in media containing sufficient sugar but reduced amounts of mineral nutrients also showed reversion to juvenile leaves.

Excised apices gave rise to simple undivided leaves when grown without a supply of carbohydrate. In media containing sugar, juvenile leaves were frequently produced for a period, but were followed by the mature leaf type.

The lateral shoots formed by feeble plants, which had been grown on unsuitable sugar concentrations, frequently bore a more juvenile leaf type than the parent rhizome. The basal segments of the rhizome always gave rise to laterals bearing juvenile leaves.

These and other results are discussed in relation to the general problem of heteroblastic development.

I am indebted to Professor C. W. Wardlaw for his continued encouragement and advice. I have to thank Mr. E. Ashby for the photographs in Pl. I and Mr. P. T. Dawes for the drawings in Text-fig. 5.

Literature Cited

Allsopp, A., 1952: Experimental and Analytical Studies of Pteridophytes. XVII. The Effect of Various Physiologically Active Substances on the Development of *Marsilea* in Sterile Culture. Ann. Bot., N.S., xvi. 165.

Ashby, E., 1948: Studies in the Morphogenesis of Leaves. 1. An Essay on Leaf Shape. New Phytol., xlvii. 153.

—— 1950: Studies in the Morphogenesis of Leaves. VI. Some Effects of Length of Day upon Leaf Shape in *Ipomaea caerulea*. Ibid. xlix. 375.

Diels, L., 1906: Jugendformen und Blütenreife im Pflanzenreich. Berlin.

Glück, H., 1911: Biologische und morphologische Untersuchungen über Wasser- und Sumpfgewächse. III. Die Uferflora, Jena.

Goebel, K., 1898: Organographie der Pflanzen. 1st ed. Jena.

—— 1908: Einleitung in die experimentelle Morphologie der Pflanzen. Leipzig.

—— 1928: Organographie der Pflanzen. 3rd ed. Jena.

Krenke, N. P., 1933–5: A Morphogenetical Analysis of the Cotton Plant. Phenogenetical Variability, 1. 419. (Russian.) Cited in Ashby, 1948.

—— 1940: Theory of Cyclic Ageing and Rejuvenescence of Plants. Moscow. (Russian.) Cited in Ashby, 1948.

Lang, W. H., 1924: On Some Deviations from the Normal Morphology of the Shoot in *Osmunda regalis*. Mem. Proc. Manchester Lit. Phil. Soc., lxviii. 53.

Lundegårdh, H., 1915: Experimentell-morphologische Beobachtungen. Flora, cvii. 433.

Mahabale, T. S., and Gorji, G. H., 1948: Some Observations on the Sporelings and Adult Plants of *Marsilea quadrifolia* Linn. Journ. Univ. Bombay, xvi. 27.

Schneider, F., 1913: Beiträge zur Entwicklungsgeschichte der Marsiliaceae. Flora, cv. 347.

Tournay, R., 1951: Études sur les hydroptéridales. 1. Le Sporophyte de Marsilea. Recherches sur *M. hirsuta* R. Br. La Cellule, liv. 163.

Troll, W., 1939: Vergleichende Morphologie der höheren Pflanzen, Erster Band, Zweiter Teil. Berlin.

Wardlaw, C. W., 1945: Experimental and Analytical Studies of Pteridophytes. VI. Stelar Morphology: The Occurrence of Reduced and Discontinuous Vascular Systems in the Rhizome of *Onoclea sensibilis*. Ann. Bot., N.S., ix. 383.

—— 1946: Experimental and Analytical Studies of Pteridophytes. VII. Stelar Morphology: The Effect of Defoliation on the Stele of *Osmunda* and *Todea*. Ibid., ix. 97.

—— 1947: Experimental and Analytical Studies of Pteridophytes. X. The Size Structure Correlation in the Filicinean Vascular System. Ibid., xi. 203.

WARDLAW, C. W., 1948: Experimental and Analytical Studies of Pteridophytes. XIII. On the Shoot Apex in a Tree Fern, *Cyathea Manniana* Hooker. Ibid., xii. 371.
—— 1949: Further Experimental Observations on the Shoot Apex of *Dryopteris aristata* Druce. Phil. Trans. Roy. Soc., ccxxxiii. 415.
—— 1952: Experimental and Analytical Studies of Pteridophytes. XVIII. The Nutritional Status of the Apex and Morphogenesis. Ann. Bot., N.S. xvi. 207.
WOLTERECK, I., 1928: Experimentelle Untersuchungen über die Blattbildung amphibischer Pflanzen. Flora, cxxiii. 30.

DESCRIPTION OF PLATE

Illustrating A. Allsopp's paper on 'Experimental and Analytical Studies of Pteridophytes. XIX. Investigations on *Marsilea*. 2. Induced Reversion to Juvenile Stages'.

Marsilea Drummondii.

FIG. 1. Three weeks after transfer from 5 per cent. glucose medium to medium without sugar. Parts to left of arrow were formed after transfer. (Nat. size.)

FIGS. 2–4. Transverse sections from plant shown in Text-fig. 1 (*a*). (× 100.) Fig. 2. At level of leaf 13 (numbered from apex). Fig. 3. At level of leaf 12. Fig. 4. At level of leaf 8. Other details in text.

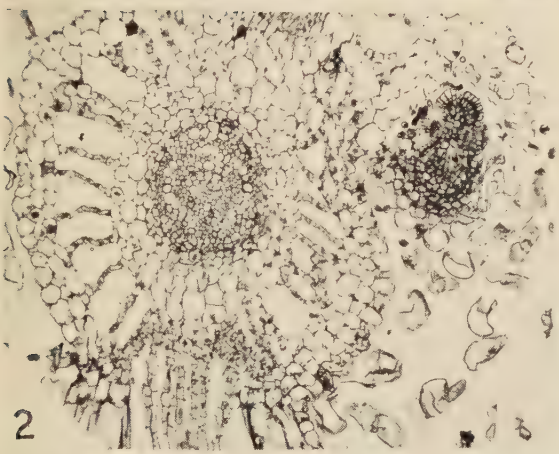
FIG. 5. After 6 months of growth on normal medium (2 per cent. glucose and standard inorganic constituents).

FIG. 6. After 6 months of growth on medium with normal glucose concentration but only $\frac{1}{5}\times$ inorganic constituents.

FIG. 7. Excised apex after 5 weeks on 2 per cent. glucose medium.

5

1



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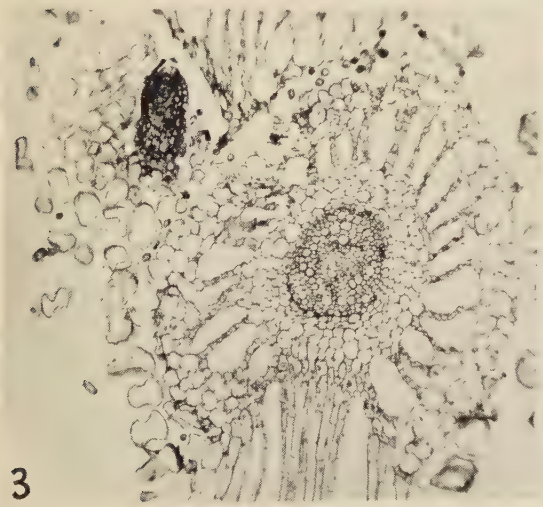

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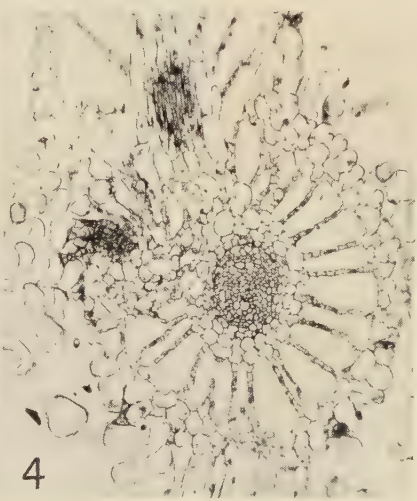

4

A Test for Non-randomness in Plant Populations

BY

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ABSTRACT

A simple test for non-randomness suitable for use in sampling plant populations is suggested. The criterion used is

$$\phi = \frac{2n_0 n_2}{n_1^2},$$

where n_i is the number of quadrats containing i shoots. A table giving significant points of ϕ for various values of N, the total number of quadrats, is provided.

The test is applied to four sets of experimental data and the results agree with tests carried out on the complete distributions. It is suggested that this type of criterion could be used in other fields where similar forms of non-randomness also exist.

I. INTRODUCTION

ARCHIBALD has discussed in two papers (1948 and 1950) the distribution of the numbers of plants of various species occurring in a number of quadrats of equal size. She has made wide use of two distributions, namely Neyman's contagious distribution and a Double Poisson series due to Thomas, for fitting to non-random plant populations that do not follow the Poisson distribution. To decide whether the distribution is of Poisson form or not it has been necessary to compute the mean and variance of the complete distribution and to test whether the latter is significantly different from the former by means of the χ^2 distribution. Archibald fits Thomas' series using the number of quadrats with no individuals and the number with one individual only. This method, though obviously not so good as a method which utilizes the whole distribution, is clearly extremely useful as it eliminates the necessity for counting the more thickly populated quadrats. The use of only the first part of the distribution for fitting suggests that it would be convenient to have a test for non-randomness similarly based on the beginning of the distribution only. The object of this paper is first to suggest a criterion on these lines, secondly to examine the theoretical side of the proposed criterion, and thirdly to give some examples of its use.

II. THE PROPOSED CRITERION

In evolving a test for non-randomness let us consider using n_0, n_1, and n_2 only where n_i is the number of quadrats with i plants. Now in a true Poisson distribution the values of n_0, n_1, and n_2 are

$$n_0 = Ne^{-\lambda}, \qquad n_1 = N\lambda e^{-\lambda}, \qquad n_2 = \tfrac{1}{2}N\lambda^2 e^{-\lambda},$$

[Annals of Botany, N.S. Vol. XVII, No. 65, January, 1953.]

where λ is the mean of the complete Poisson distribution and N is the total number of quadrats observed. Hence if we take

$$\phi = \frac{2n_0 n_2}{n_1^2}, \tag{1}$$

we see that in the case of a true Poisson distribution the value of ϕ is unity.

In experiments on the numbers of larvae in fixed areas, described by Neyman (1939), it is stated that the attempts to fit the Poisson distribution 'failed almost invariably with the characteristic feature that, as compared with the Poisson Law, there were too many empty plots and too few plots with only one larva'. Feller (1943) has shown that this characteristic is bound to arise whenever the material under observation is not quite random so that the compound Poisson law applied instead of the simple law. Archibald (1948) remarks that many of the writers on plant populations had noticed this feature.

Now it can be seen from (1) that when we have the kind of non-randomness envisaged above the criterion ϕ will be greater than unity, and hence a test for the non-randomness or otherwise of an expected Poisson distribution might reasonably be based on ϕ. This test would be of great value as it would eliminate any errors due to counting in the quadrats of high density and save a certain amount of time in computation.

III. The Theoretical Distribution of ϕ

It is necessary to study, in the first place, the distribution of ϕ when the true distribution is of Poisson type. By writing ϕ in a slightly different form the true moments of the criterion have been obtained, but they are extremely lengthy and will not be given here. However, by using an approximate method it can be shown that the first two moments of ϕ are

$$\mu_1 = 1 + \frac{3}{Np_1}, \tag{2}$$

$$\mu_2 = \frac{1}{N}\left[\frac{1}{p_0} + \frac{4}{p_1} + \frac{1}{p_2}\right], \tag{3}$$

where p_0, p_1, and p_2 are the probabilities of getting 0, 1, and 2 plants per quadrat respectively under the Poisson law. The derivation of these moments is outlined in the Appendix.

We have already seen that we are interested in detecting significantly large values of ϕ and hence we may reasonably take values of ϕ greater than

$$\mu_1(\phi) + 2\sqrt{\{\mu_2(\phi)\}} \tag{4}$$

as establishing a significant discrepancy from the Poisson law. The significance level thus found will be about the 5 per cent. level, which is the common one in use. In Table I the values of (4) are given for various values of N, the total number of quadrats observed. It is clear that the values of (2) and (3)

which depend on p_0, p_1, and p_2 are related to λ, the Poisson parameter corresponding to the complete distribution. This would normally have to be estimated from the mean of the whole distribution. However, to obviate this another variable R is given below λ in Table I, where

$$R = \frac{n_0+n_1+n_2}{N} \times 100, \tag{5}$$

i.e. it is the percentage of the distribution falling in the first three groups. As there is a unique relationship between λ and R the use of R is equivalent to estimating λ from the first three groups only. Hence to carry out the test only four quantities are needed, namely, n_0, n_1, n_2, and N. From these four ϕ may be calculated from (1) and R from (5). Entering Table I with R and N we can see whether our calculated value for ϕ exceeds the one given. If it does so there is definite evidence of non-randomness and the Poisson law is inadequate to describe the data.

TABLE I

Significant Points for ϕ

	λ	0·5	1·0	1·5	2·0	2·5
	R	99	92	81	68	54
N	50	2·70	2·40	2·46	2·66	2·98
	100	2·16	1·95	1·99	2·13	2·34
	200	1·80	1·66	1·68	1·77	1·92
	300	1·65	1·53	1·55	1·62	1·74
	400	1·55	1·46	1·47	1·54	1·63
	500	1·49	1·41	1·42	1·48	1·56

The figure in the main body of the table is the mean of ϕ plus twice its standard error.

IV. Experimental Results

We will now discuss some examples of the use of the proposed criterion. First we give two examples from Archibald (1948). Table II gives the distribution of the number of shoots per quadrat for *Triglochin maritima*.

TABLE II

Frequency Distribution of Triglochin maritima

No. of shoots per quadrat	0	1	2	3	4	5	Total
No. of quadrats	39	23	17	13	4	4	100

For these figures ϕ is equal to 2·51, R is 79, and N is 100. From Table I the significant point for ϕ is approximately 2·00, and thus there is considerable evidence of non-randomness and the hypothesis of randomness should be discarded.

In Table III the frequency distribution of *Armeria maritima* is given. For this case we have ϕ equal to 38, R equal to 75, and N is 100.

TABLE III

Frequency Distribution of Armeria maritima

No. of shoots per quadrat	0	1	2	3	4	5	6	7	8	9	10	Total
No. of quadrats . .	57	6	12	5	5	5	7	1	—	1	1	100

From Table I the significant point for ϕ is approximately 2·06, and we have thus got an extremely significant result. As Archibald has pointed out, Neyman's contagious distribution provides a good fit for this bi-modal distribution.

As a third example we take the distribution of *Carex flacca* from Archibald (1950). The figures are given in Table IV, and we find that ϕ is equal to 2·52, R is 79, and N is 500. From Table I the significant point for ϕ is about 1·43, and hence our observed value of ϕ is highly significant, showing once again evidence of non-randomness.

TABLE IV

Frequency Distribution of Carex flacca

No. of shoots per quadrat . .	0	1	2	3	4	5	6	7	8	Total
No. of quadrats . .	181	118	97	54	32	9	5	3	1	500

As a last example we take the distribution of the numbers of noxious weed seeds in quarter-ounce samples of *Phleum pratense* seeds. The data are taken from Snedecor (1946) and are given in Table V. The value of ϕ is 0·54, R is 47,

TABLE V

Frequency Distribution of Noxious Weed Seeds

No. of noxious weed seeds .	0	1	2	3	4	5	6	7	8	9	Total
No. of samples . .	3	17	26	16	18	9	3	5	—	1	98

and N is 98. From Table I the value of ϕ is clearly not significant and there is no evidence of lack of randomness. In actual fact a Poisson distribution fitted to the complete data gives excellent agreement which is borne out by a χ^2 test for goodness of fit.

V. CONCLUSIONS

The examples given above have illustrated how the proposed technique may be used to determine whether the underlying basis of the distribution is of Poisson form or whether there is some form of non-randomness present. In all four cases an examination of the complete distribution shows the results

based on ϕ to be well founded. It will be noticed from Table I that the significant points for ϕ appear to be least when the value of λ, or mean density of plants per quadrat or sample, is unity. This gives a rough indication of the size of quadrat that it would be best to use to carry out an analysis of this form. We may note in passing that for a Poisson series with a mean of unity n_0 would be equal to n_1.

The proposed method possesses the merit of simplicity and obviates the necessity for counting the quadrats of high density and hence eliminates some possible counting errors that might otherwise creep in. Further, if the method of fitting for a non-random distribution is to be based on the beginning part of the distribution only, it is clearly very inconvenient to have to obtain the whole distribution to carry out a test for randomness.

The method has obvious applications in other fields. For instance, in studying the distribution of yeast cells in counts with a haemacytometer or the numbers of earthquakes occurring in successive equal intervals of time at a certain place. Another use that has been found is to modify ϕ and base it on n_1, n_2, and n_3, and then apply the result to figures relating to the numbers of persons in a house succumbing to a certain disease in an epidemic. In this case n_0, the number of houses with no cases, is rarely known but the other values are known accurately. To judge whether the value of the modified ϕ is significant or not only an approximate estimate of the total number of houses is needed and the value obtained indicates that there is definite evidence of departure from the Poisson form.

Appendix

Derivation of Moments of ϕ

We define $$\phi = \frac{2n_0 n_2}{n_1^2} = 2\frac{(m_0+\delta m_0)(m_2+\delta m_2)}{(m_1+\delta m_1)^2},$$

where m_i is the true population frequency and δm_i is the deviation from it due to random sampling fluctuations. Thus $m_i+\delta m_i = n_i$.

We require to find the first two moments of ϕ under the assumption that a sample of size N has been randomly and independently drawn from some population where p_i is the probability that an individual falls in the ith cell.

From above

$$\phi = \frac{2m_0 m_2}{m_1^2}\left(1+\frac{\delta m_0}{m_0}\right)\left(1+\frac{\delta m_2}{m_2}\right)\left(1+\frac{\delta m_1}{m_1}\right)^{-2}$$

$$= \frac{2m_0 m_2}{m_1^2}\left(1+\frac{\delta m_0}{m_0}+\frac{\delta m_2}{m_2}-2\frac{\delta m_1}{m_1}+\frac{\delta m_0\,\delta m_2}{m_0 m_2}-\right.$$

$$\left.-2\frac{\delta m_0\,\delta m_1}{m_0 m_1}-2\frac{\delta m_1\,\delta m_2}{m_1 m_2}+3\frac{\delta m_1^2}{m_1^2}\right),$$

neglecting all powers of $\delta m_i/m_i$ higher than the second.

Now for a Poisson population $2m_0 m_2/m_1^2$ is unity and the first moment of ϕ, or mean value of ϕ in repeated sampling, is

$$\mathscr{E}(\phi) = 1 - \frac{Np_0p_2}{N^2p_0p_2} + 2\frac{Np_0p_1}{N^2p_0p_1} + 2\frac{Np_1p_2}{N^2p_1p_2} + 3\frac{Np_1(1-p_1)}{N^2p_1^2}$$

$$= 1 + \frac{3}{Np_1} \quad \text{after simplification.}$$

To obtain the expression we use two relationships:

$$\mathscr{E}(\delta m_i\, \delta m_j) = -Np_ip_j \quad (i \neq j)$$

$$\mathscr{E}(\delta m_i^2) = Np_i(1-p_i).$$

For a proof of these relationships see, for example, David (1949). For the second moment we need the mean value of ϕ^2 in repeated sampling, and by following a similar procedure to that used for ϕ we have

$$\mathscr{E}(\phi^2) = 1 + \frac{1}{N}\left[\frac{1}{p_0} + \frac{10}{p_1} + \frac{1}{p_2}\right].$$

Now the true second moment about the mean of ϕ is

$$\mu_2(\phi) = \mathscr{E}(\phi^2) - \{\mathscr{E}(\phi)\}^2$$

$$= 1 + \frac{1}{N}\left[\frac{1}{p_0} + \frac{10}{p_1} + \frac{1}{p_2}\right] - \left[1 + \frac{6}{Np_1}\right]$$

$$= \frac{1}{N}\left[\frac{1}{p_0} + \frac{4}{p_1} + \frac{1}{p_2}\right].$$

These expressions have been utilized to obtain the significant points given in Table I.

Literature Cited

Archibald, E. E. A., 1948: Plant Populations, I. A New Application of Neyman's Contagious Distribution. Ann. Bot., N.S., xii. 221.

—— 1950: Plant Populations, II. The Estimation of the Number of Individuals per Unit Area of Species in Heterogeneous Plant Populations. Ibid., xiv. 7.

David, F. N., 1949: Probability Theory for Statistical Methods, p. 143 (Cambridge University Press).

Feller, W., 1943: On a General Class of 'Contagious' Distributions. Ann. Math. Stat., xiv. 389.

Neyman, J., 1939: On a New Class of 'Contagious' Distributions applicable in Entomology and Bacteriology. Ibid., x. 35.

Snedecor, G. W., 1946: Statistical Methods, p. 441 (The Iowa State College Press).

Rhizomorph Behaviour in *Armillaria Mellea* (Vahl) Quél.

I. Factors controlling Rhizomorph Initiation by *A. Mellea* in Pure Culture

BY

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With Plate II

ABSTRACT

Experiments on rhizomorph initiation by *Armillaria mellea* in agar plate culture are described, showing that production of rhizomorph initials is controlled by a nutritional mechanism.

I. INTRODUCTION

THE significance of the rhizomorph in the evolution of root-infecting fungi calls for further experimental study of rhizomorph behaviour. Any investigation of the host/parasite relationships in which the rhizomorph is involved must logically be preceded by a study of the growth and physiology of the rhizomorph in pure culture; the first results of such a study are presented in this paper. As this aspect of the work has been concerned solely with the growth of *Armillaria mellea* by itself in pure culture on the agar plate, no attempt will be made at this point to review work and current theories on the function of the rhizomorph in the infection of the host root, especially as this has recently been done elsewhere (Garrett, 1951).

The work described below has been primarily concerned with factors controlling the *initiation* of rhizomorphs in pure culture rather than with those controlling subsequent growth in length, although some information on the latter has necessarily been obtained during the course of these experiments. No such intensive investigation into the physiological mechanism of rhizomorph initiation has yet been reported by any other worker; under some conditions at least, many more rhizomorph initials are produced than subsequently develop, and these cannot be seen unless a careful search for them is made under conditions appropriate for detection. It is impossible, for instance, to count rhizomorph initials in an agar slant culture in a tube, or even to make a reliable count of older rhizomorphs; under such conditions the best that can be done is to make a visual assessment of rhizomorph development and to express it by means of a grading, as was done by Hamada (1940).

[Annals of Botany, N.S. Vol. XVII, No. 65, January, 1953.

An additional incentive to the undertaking of this work was provided by the recognition of an interesting parallel between a carbohydrate/nitrogen interaction affecting the development of rhizomorphs by *A. mellea*, discovered by Hamada (1940), and an apparently similar interaction governing production of sclerotia by *Helicobasidium purpureum* (Garrett, 1949). In the technique employed by Garrett experiments took 7 months to complete, and attempts to devise a more rapid technique have been unsuccessful. Experiments on initiation of rhizomorphs by *A. mellea* can be completed within 2 weeks, so that this fungus is obviously a more suitable organism for further investigation of this carbohydrate/nitrogen interaction than is *H. purpureum*. The results already obtained by Hamada have been confirmed, elucidated, and further extended, and *A. mellea* is eminently suitable for further work on this problem. This confirmation and extension of Hamada's findings strengthen the tentative conclusion recently advanced (Garrett, 1951) that there is a close analogy, morphological, physiological, and even functional, between the rhizomorph and the sclerotium.

II. General Methods

The isolate of *Armillaria mellea* employed throughout these experiments was from the culture collection of the Cambridge Botany School and had been isolated in 1946 from *Daphne mezereum* growing in the Cambridge garden of Professor F. T. Brooks. This isolate was very consistent in its rhizomorph production. It appears from the literature that the great majority of *A. mellea* isolates produce rhizomorphs freely on a suitable agar substrate, though Benton and Ehrlich (1941), in a study of twenty-seven isolates obtained from *Pinus monticola*, reported that some of their isolates 'developed a very thin mycelium with no rhizomorphs' on malt agar, but that the same isolates produced rhizomorphs when growing on wood blocks.

All experiments were incubated at 25° C., in view of the finding of Reitsma (1932) that this is the optimum temperature for growth of *A. mellea*. The optimum reaction of the medium was found by Reitsma, and confirmed by Benton and Ehrlich (1941), to be pH 5; the majority of the agars employed in the following experiments had pH values in this region. Other details of the methods employed in this work will be furnished in their context below. In the statistical analysis of experimental results use was made of the statistical tables of Fisher and Yates (1949); full analyses of variance were worked out for all multiple factor experiments, but the citation of the statistical values derived therefrom has been restricted to the essential minimum.

III. Experiments on Nutritional Requirements for Growth of *A. mellea*

Experiment 1. In view of the fact that this study of rhizomorph behaviour would be chiefly concerned with nutrition, it was obviously essential to determine the nutritional requirements of *A. mellea* at the outset. For this purpose the fungus was grown in liquid culture in medicine bottles of some 180 ml.

capacity; 10 ml. culture solution were allotted to each bottle so as to give a suitably shallow layer of liquid medium when the bottle was stacked flat on its wider side. In this experiment the basal medium employed throughout contained 2 per cent. dextrose, 0·1 per cent. potassium phosphate, 0·05 per cent. crystalline magnesium sulphate, and 0·2 mg. per litre ferric chloride. Three nitrogen sources, sodium nitrate, ammonium tartrate, and peptone, were tested at 4 equivalent concentrations of nitrogen, 0, 0·01, 0·02, and 0·04 per cent. Ammonium tartrate was selected as likely to be the most suitable source of ammonium nitrogen in view of recent work by Brian, Curtis, and Hemming (1947) on uptake of ammonium salts by fungi. In this comparison of nitrogen sources growth factors were supplied at a standard rate as follows: thiamin at the rate of 100 gamma (0·1 mg.) per litre, and biotin at the rate of 75 gamma (0·075 mg.) per litre. The effect of presence or absence of growth factors, individually and together, was tested in the presence of each nitrogen source at the concentration of 0·02 per cent. N. Three bottles were provided for each of the twenty-one nutritional series in this experiment, making 63 bottles in all; each bottle was inoculated with a No. 1 cork borer disc (4 mm. diameter) of agar taken marginally from a colony of *A. mellea* growing on plain water agar. After inoculation the bottles were stacked in the 25° C. incubator and left there for 27 days; after this period, the mycelial pads were removed from the bottles, washed, and then dried to constant weight in an oven at 80° C. The mycelial pads from the three replicate bottles of each series were pooled, being washed, dried, and weighed together. Mycelial dry weights are given in Table I.

TABLE I

Mycelial Dry Weights in mg.

	% nitrogen.			
	0·00	0·01	0·02	0·04
As nitrate	2·5	2·3	2·5	3·0
,, tartrate . . .	2·0	68·2	61·0	52·0
,, peptone . . .	2·9	68·5	154·0	259·5

	Thiamin+ biotin.	Thiamin only.	Biotin only.	No growth factors.
As nitrate	2·5	2·1	2·7	2·0
,, tartrate . . .	61·0	128·0	10·0	15·5
,, peptone . . .	154·0	268·5	36·5	53·0

These figures clearly show that this particular isolate of *A. mellea* is unable to utilize nitrate nitrogen. This result has been confirmed in a further and equally conclusive experiment, the data for which need not be given. Failure to grow on a nitrate source of nitrogen cannot be ascribed to the lack of any essential growth factor other than thiamin, because good growth was made on ammonium tartrate with thiamin as the sole growth factor provided. It is necessary to qualify these results, however, as applying only to the particular

isolate of *A. mellea* used here, because Reitsma (1932) obtained an appreciable growth (31 mg. mycelial dry weight) of *A. mellea* in liquid culture with potassium nitrate as the source of nitrogen; better growth was obtained with ammonium sulphate (64 mg.), and the best growth of all (214 mg.) with peptone. Reitsma listed the nitrogen sources investigated by him in decreasing order of availability as follows: peptone, asparagin, glycocoll, ammonium tartrate, ammonium sulphate, ammonium chloride, and potassium nitrate. There is no serious discrepancy between Reitsma's results on utilization of nitrate nitrogen and those reported above in Table I; nitrates are evidently the poorest sources of nitrogen for *A. mellea*, and it is not surprising that some strains of this fungus should be able to make a limited use of nitrate whilst others may completely have lost the ability to do this. A close parallel may be found in those fungi having a 'partial' requirement for a particular growth factor, indicating that rate of synthesis is inadequate for the needs of growth (Hawker, 1950, p. 77). Such a partial or complete loss by different strains of *A. mellea* of the ability to reduce nitrates, and hence to utilize nitrate nitrogen, is quite consistent with the evolution of this fungus as a specialized root parasite (Garrett, 1950).

Concerning growth factor requirements, the figures show that, with ammonium tartrate as nitrogen source, little growth was made in the absence of thiamin; the greater growth made in the absence of thiamin when peptone was the nitrogen source may be ascribed to the thiamin content of the peptone. At the concentration employed in this experiment (75 gamma per litre) biotin has exerted a markedly depressing effect on mycelial growth, as shown by comparison of growth with thiamin in the presence and absence of biotin.

Experiment 2. This experiment was set up in an attempt to confirm and elucidate the depressing effect upon growth of too high a concentration of biotin, as shown in the previous experiment, to find out whether a lower concentration of biotin was essential or beneficial for growth and to determine the optimal concentration of thiamin. The technique used was the same as in the previous experiment; the basal solution contained 2 per cent. dextrose, 0·01 per cent. nitrogen as ammonium tartrate, 0·1 per cent. potassium phosphate, 0·05 per cent. crystalline magnesium sulphate, and 0·2 mg. per litre ferric chloride. With thiamin constant at 100 gamma per litre, biotin concentrations of 80, 40, 20, 10, 5, and 0 gamma per litre were tested: with biotin constant at 5 gamma per litre, thiamin concentrations of 100, 50, 25, 12·5, and 0 gamma per litre were tested. Four replicate bottles were set up for each of the 11 experimental series, making 44 bottles in all; for inoculum, 4 mm. agar discs were taken marginally from a colony of *A. mellea* growing on plain water agar. The bottles were incubated at 25° C., and mycelial dry weights determined after 39 days' growth (Table II).

Even at 80 gamma per litre, biotin has not produced a depression in growth comparable with that in the previous experiment. It is possible that the depressing effect of biotin is exerted particularly in the earlier phases of mycelial growth and that, in the longer growth period allowed for this

TABLE II

Mycelial Dry Weights in mg.

With thiamin constant at 100 gamma.		With biotin constant at 5 gamma.	
Biotin, 80 gamma	–130	Thiamin, 100 gamma	–155·5
,, 40 ,,	–110	,, 50 ,,	–127
,, 20 ,,	–122	,, 25 ,,	–141
,, 10 ,,	–117	,, 12·5 ,,	– 96
,, 5 ,,	–155·5	,, 0 ,,	– 11
,, 0 ,,	–142·5		

experiment (39 days as against 27 days in expt. 1), growth at the higher biotin concentrations caught up with that at the lower. It can certainly be concluded from this experiment that biotin is not essential, or even beneficial, for growth. Concerning the effect of thiamin concentration, the figures suggest that the optimal concentration of thiamin is reached at 25 gamma per litre, but that growth is still maximal at 100 gamma per litre. No further experiments were carried out on growth factor requirements, inasmuch as the information essential for the main experiments on rhizomorph behaviour had been obtained, viz. that satisfactory growth could be assured on a synthetic medium containing ammonium tartrate or peptone as a source of nitrogen, together with thiamin at the rate of 25–100 gamma per litre.

IV. Experiments on Rhizomorph Initiation by *A. mellea* in Agar Plate Cultures

These experiments were carried out in 9-cm. diameter Petri dishes poured with 20 ml. agar, to give a depth of 3 mm. agar. Dishes were inoculated centrally in some experiments; in others, two or four inoculum discs were spaced so as to give a minimum of interference with one another. Except where otherwise stated, standard inoculum discs, 4 mm. in diameter, were obtained by cutting around the mycelial margin of a colony with a sterile No. 1 cork borer; particular care was always taken to exclude segments of rhizomorphs, or of rhizomorph branches, from such inoculum discs, inasmuch as the experiments were designed to elucidate the mechanism of rhizomorph initiation from the unorganized mycelium. Cultures were grown at 25° C. throughout the experiments.

The behaviour of young cultures arising from such an inoculum disc laid on a plate of fresh agar was remarkably consistent throughout these experiments; on any agar suitable for rhizomorph production the sequence of events after inoculation of the plate was as follows. Some 24 hours or more after inoculation a fringe of hyphae commences to grow out from the margin of the inoculum disc over and through the fresh agar; after 7 days on most agars a circle of rhizomorph initials becomes visible to the naked eye around the margin of the original inoculum disc (Pl. II, Fig. 1). With occasional exceptions, the rhizomorphs thus formed grow into the agar and then travel parallel

and adjacent to the glass bottom of the dish; direction of growth is not controlled geotropically, because this habit of growth invariably occurs whether Petri dishes are incubated 'upside down' (as was the routine procedure in these experiments) or 'right side up'. Numbers of rhizomorph initials can thus be conveniently counted, and subsequent growth of rhizomorphs observed, through the glass bottom of the dish by inspection with reflected light under a binocular dissecting microscope, using a magnification $\times 25$.

Under certain circumstances rhizomorph initials may be formed but fail to make any appreciable growth, being apparently unable to compete with the unorganized mycelium. Normally, however, the young rhizomorphs, which start growth later than the unorganized mycelium, grow much more quickly than the hyphae comprising this mycelium, and soon pass beyond its margin; from the rhizomorphs, which later branch, fringing mycelium grows out into the surrounding agar, and this imparts a deeply lobed appearance to the whole colony of rhizomorphs and fringing mycelium (Pl. II, Fig. 2).

During this investigation no new rhizomorphs have ever been observed to arise in undisturbed plate colonies apart from those initiated around the original inoculum disc. The original object of this investigation was to elucidate the mechanism controlling rhizomorph initiation; it soon became apparent that any hypothesis put forward must explain the inhibiting effect apparently exercised by existing rhizomorphs over the inception of fresh initials in their own fringing mycelium. At the outset of this work rhizomorphs and unorganized mycelium were viewed as alternative and possibly competing phases in the vegetative existence of *A. mellea*; as the investigation progressed, it became more appropriate to regard the rhizomorph with its fringing mycelium as a single organ; indeed, the root of a higher plant with its root-hairs appears to offer a physiological analogy that goes deeper than the mere superficial resemblance.

The second most significant observation made during this study relates to the remarkable difference between the respective growth rates of rhizomorphs and unorganized mycelium; the maximum mean growth rate observed for rhizomorphs was 25 mm. per week, whereas the maximum for unorganized mycelium was only 4–5 mm., i.e. rhizomorphs grew 5–6 times as rapidly as did unorganized mycelium. This observation strengthens the suggestion advanced elsewhere (Garrett, 1951) that the rhizomorph is an organ primarily concerned with the translocation of nutritional reserves from one substrate (the 'food-base') to another, and that, in the case of such a root parasite as *A. mellea*, speed of translocation is equal in importance to distance and efficiency of translocation. The rapid growth of rhizomorphs either ectotrophically along the outside of a host root, or alternatively along a line of mechanical weakness between the bark and the wood, is more likely to overwhelm or to evade such active defence mechanisms as the host root may possess than would a slower invasion of the fungus concentrated at a single point. Nevertheless, if the rhizomorph and its fringing mycelium be viewed as a single organ, for which view experimental evidence will be brought

forward later, then the mechanism for rapid consolidation of territory won either from a dead substrate or from living host tissues is also seen to be available.

1. *The nutritional requirements for rhizomorph initiation*

Experiment 1. The object of this experiment was to investigate the effect of carbohydrate concentration, both in the inoculum disc and in the fresh growth medium, upon the number of rhizomorph initials formed around the inoculum disc. Colonies for the production of inoculum discs were accordingly grown on a series of four media, containing 0, 1, 2, and 4 per cent. dextrose, respectively, in addition to basal carbohydrate and nitrogen, &c., supplied in the form of 0·5 per cent. malt extract+0·15 per cent. peptone to all four media; this experiment was set up before results were available from the two experiments described in the preceding section, so that a basal addition of malt extract was made in order to supply possibly essential growth factors. From the colonies on these four media, standard 4 mm. discs of inoculum were taken from the margins with a sterile No. 1 cork borer, and used to inoculate the same series of media, to give all sixteen possible combinations of inoculation and growth media. As this experiment was designed to provide information only about the *number* of rhizomorphs initiated around each inoculum disc (and not about their subsequent growth in length), each plate was inoculated at four points. The four inoculum discs were spaced on two diameters at right angles, each disc being equidistant from the centre of the plate and from the margin; the process of inoculation was facilitated by the use of a ten-point inoculating needle, previously devised for serial (not simultaneous) multiple inoculation of plates (Garrett, 1946). Three plates were inoculated for each of the inoculum disc/growth medium combinations, so that the numbers of rhizomorph initials given in Table III are means of 12 counts for each series.

TABLE III

No. of Rhizomorph Initials around Inoculum Disc

	Per cent. of supplementary dextrose: in growth medium.				
In inoculum.	0	1	2	4	Mean (±1·525)
0	1	39	27	23	23
1	5	21	23	19	17
2	7	24	27	19	19
4	10	21	26	21	19
Mean (±1·525)	6	26	26	20	—

Standard error for individual treatment means = 3·05.

These figures show that the carbohydrate contained in 0·5 per cent. malt extract (dry matter *c.* 75 per cent.), without supplementary dextrose, was insufficient for maximum production of rhizomorph initials; an analysis of variance performed on the above data indicates that rhizomorph production

on the growth medium without supplementary dextrose was very significantly lower ($P < 0{\cdot}001$) than that on the medium with 1 per cent. supplementary dextrose. The number of initials was not increased by a further rise in supplementary dextrose to 2 per cent., and it was significantly depressed ($P = 0{\cdot}01$) by the 4 per cent. addition of dextrose to the growth medium; this concentration of supplementary dextrose was evidently supra-optimal.

The statistical analysis further indicates the occurrence of a significant interaction ($P = 0{\cdot}01$) between dextrose concentration in the inoculum disc and that in the growth medium. Further examination of the individual treatment means given in Table III in the light of their standard error (3·05) shows quite clearly that this interaction is comprised of two effects. Firstly, on the growth medium with no supplementary dextrose there is a significant increase ($P = 0{\cdot}05$) in mean number of initials from only one with no supplementary dextrose in the inoculum up to 10 with 4 per cent. supplementary dextrose in the inoculum; on this carbohydrate-deficient medium the inoculum disc has made a significant contribution to the dextrose required for production of rhizomorph initials. Such an effect is not seen on the growth media containing 1 per cent. or more supplementary dextrose. Secondly, the dextrose content of the inoculum disc has influenced the number of initials in the reverse way on the growth medium containing 1 per cent. supplementary dextrose; 39 initials (by far the highest number in the whole experiment) were produced by the combination of no supplementary dextrose in the inoculum with 1 per cent. in the growth medium. This effect is very highly significant ($P < 0{\cdot}001$).

Other evidence also indicates that production of rhizomorph initials is maximal when an inoculum disc of minimal nutrient content is laid on a fresh agar substrate containing not less than 1 per cent. dextrose. Thus the maximum production of initials is given by inoculum discs taken from colonies growing on plain water agar. This may be connected with the wider spacing of the hyphae in the mycelium on such a poor substrate; the reduced competition between individual hyphae would result in a more rapid rise in carbohydrate concentration within such individual hyphae when the inoculum disc is laid on fresh agar containing 1 per cent. dextrose than if hyphae were more densely crowded in the inoculum disc.

Experiment 2. This experiment was designed to confirm and elucidate results on the interaction of dextrose and peptone in their effects upon production and growth of rhizomorphs, previously reported by Hamada (1940). Working over the range 0–8 per cent. dextrose and 0–8 per cent. peptone, giving a maximum nitrogen content of *c.* 1·2 per cent.[1] at the highest level, Hamada found that increasing the nitrogen content of the medium above a certain point depressed rhizomorph development at the lower carbohydrate concentrations; as the carbohydrate concentration was raised, however, the optimum nitrogen concentration rose with it, and the depressing effect of higher nitrogen concentrations disappeared at the higher carbohydrate levels.

[1] Hamada gives the nitrogen content of his peptone as *c.* 15 per cent.

Hamada's experiment was carried out on agar slants in tubes, and his assessment of rhizomorph production was therefore based on a visual estimate of total growth; under such circumstances reliable counts neither of initials nor even of older rhizomorphs could be made.

The basal medium employed for this experiment contained 0·1 per cent. potassium phosphate, 0·05 per cent. crystalline magnesium sulphate, 0·2 mg. per litre ferric chloride, and 100 gamma per litre thiamin. Dextrose concentrations were 1 and 2 per cent., in combination with peptone concentrations of 0·15, 0·3, 0·6, 1·2, 2·4, and 4·8 per cent.; the nitrogen content of the peptone employed in all experiments reported in this paper was 13·2 per cent., so that the corresponding nitrogen concentrations of these media ranged from 0·02 to 0·64 per cent. Plates were inoculated at four points, as in expt. 1, with 4 mm. marginal inoculum discs taken from colonies on 0·05 per cent. malt extract+0·15 per cent. peptone agar; 3 plates were inoculated in this way for each of the 12 dextrose series, so that numbers of rhizomorph initials given in Table IV are means of 12 counts for each series.

TABLE IV

No. of Rhizomorph Initials around Inoculum Disc

	% N.					
	0·02	0·04	0·08	0·16	0·32	0·64
1% dextrose . . .	11	17	21	22	17	16
2% dextrose . . .	11	17	24	22	23	24

Standard error for individual treatment means = 1·06.

In both dextrose series the numbers of rhizomorph initials increased with rising nitrogen content to 0·08 per cent., and it appears that concentrations of nitrogen lower than this were suboptimal for rhizomorph initiation. There was a fully significant decrease ($P = 0{\cdot}01$) in numbers of rhizomorph initials produced at the two highest nitrogen levels in the 1 per cent. dextrose series, but this effect is not seen at the higher dextrose concentration. Nevertheless, such an effect on *numbers* of rhizomorphs would be quite insufficient to account for the results reported by Hamada. The design of the present experiment did not lend itself to accurate and prolonged recording of rhizomorph growth in length inasmuch as each plate had been inoculated at four separate points; nevertheless, it was quite obvious that outward growth of rhizomorphs from the inoculum disc was depressed at the higher nitrogen levels. A satisfactory quantitative estimate of this depression was obtained, however, by recording numbers of rhizomorphs that had grown out beyond the mycelial margin of the colony, to produce 'lobing' of the margin, after 16 days' incubation of the plates at 25° C.; the figures given in Table V are means of 12 colony counts for each series.

By comparing these figures with those for numbers of rhizomorph initials produced in the same series of plates (Table IV) it is evident that either a

TABLE V

No. of Rhizomorphs growing out beyond Colony to produce 'Lobing' of Colony Margin

	% N.					
	0·02	0·04	0·08	0·16	0·32	0·64
1% dextrose . . .	4	10	12	7	1	0
2% dextrose . . .	4	7	12	12	12	1

Standard error for individual treatment means = 0·74.

deficiency or an excess of nitrogen will check the outwards growth of rhizomorphs relative to that of the unorganized mycelium. In the extreme case, a deficiency of nitrogen leads to a complete suppression of rhizomorph initials soon after they become visible, as has been observed in some of the later experiments. The depression of rhizomorph growth at supra-optimal concentrations of nitrogen incidentally illustrates a point of general physiological interest, viz. that the tolerance of unorganized mycelium for adverse conditions is wider than that of the rhizomorphs. These effects of peptone nitrogen on the rate of growth of rhizomorphs constitute merely a particular case of the physiological generalization expressed by Hawker (1950, pp. 134 and 162) to the effect that the range of nutritional and other environmental conditions permitting sporulation is narrower than that for mycelial growth; both the rhizomorph and the sclerotium may be considered as forms of asexual reproduction, and hence closely analogous to asexual sporulation.

From the results of the above experiment as a whole, the dextrose-peptone interaction observed by Hamada can be interpreted primarily as an effect upon rhizomorph growth in length.

Experiment 3. Following upon the previous experiment, it seemed desirable to investigate further the effect of this carbohydrate-nitrogen interaction upon *initiation* of rhizomorphs, over the lower concentration ranges in which such an effect was likely to be most pronounced. A secondary objective of this experiment was to determine the minimum concentrations of carbohydrate and nitrogen, respectively, that would permit rhizomorph initiation. The same basal medium was employed as in expt. 2; dextrose levels were 0·125, 0·25, 0·5, and 1 per cent., and peptone concentrations were adjusted to give series containing 0·0025, 0·005, 0·01, and 0·02 per cent. nitrogen respectively. The highest concentrations of dextrose and nitrogen, respectively, employed in this experiment were thus the lowest employed in expt. 2. In order to eliminate any appreciable contribution of nutrients from the inoculum, the 4 mm. marginal discs were taken from colonies on plain water agar, 28 days old at 25° C. and *c.* 34 mm. diameter. Only two inoculum discs per plate were permitted for this experiment, each being equidistant from the centre of the plate and from the margin; this precaution was taken in order to avoid any possible competition for nutrients between the young colonies at the lower nutrient concentrations. Three plates were inoculated in this way for

each of the 16 dextrose/peptone series, so that the figures given in Table VI are means of 6 counts for each series.

TABLE VI

No. of Rhizomorph Initials around Inoculum Disc

	% N.			
% dextrose	0·0025	0·005	0·01	0·02
0·125	1·7	1·7	0·8	0·2
0·25	3·0	5·3	2·8	2·3
0·5	5·8	7·7	8·3	13·0
1·0	4·0	8·7	11·3	22·7

Standard error for individual treatment means = 1·35.

An analysis of variance performed on the above data indicates that the interaction between carbohydrate and nitrogen is very highly significant ($P < 0{\cdot}001$). The depressing effect of supra-optimal nitrogen concentration is seen only at the two lower dextrose levels; with rising dextrose concentration the optimum concentration of nitrogen for maximum production of rhizomorph initials also rises. It must be emphasized that the figures given in Table VI refer specifically to numbers of rhizomorph initials, and not to numbers of fully developed rhizomorphs; at the lower nutrient levels many of the initials failed to develop farther. Such failure of initials to continue growth can be ascribed to competition between the young initials and the unorganized mycelium, which has the advantage of an earlier start in growth (see introduction to section IV).

Another feature of particular interest emerges from these results when the mean figure of 22·7 rhizomorph initials for the medium with 1 per cent. dextrose+0·02 per cent. nitrogen in Table VI is compared with the mean figure of 11 for the same medium in Table IV. These two media were identical in composition; it is possible, though not likely, that interference in nutrient absorption due to competition between colonies was responsible for the lower number of initials formed in expt. 2, in which there were 4 colonies per plate. A more probable cause of this difference lies in the fact that whereas in expt. 2 the inoculum discs were taken from colonies on 0·5 per cent. malt extract+0·15 per cent. peptone agar, in the present experiment inoculum discs were taken from colonies growing on plain water agar. In expt. 1 it was shown that, for a growth medium containing *c.* 1 per cent. dextrose, a decrease in nutrient content of the inoculum disc gave an increase in the number of rhizomorph initials.

2. *The mechanism inhibiting production of new rhizomorph initials in established colonies*

In the introduction to this section the normal development of a colony of *A. mellea* on a plate of agar suitable for rhizomorph production has been

described; no new rhizomorph initials have yet been observed to arise within an undisturbed colony of growing rhizomorphs and fringing mycelium. In the experiments now to be described an attempt was made to elucidate the mechanism controlling production of new initials within an established colony, by disturbing in various ways the normal course of colony development.

Experiment 1. In a preliminary experiment the rhizomorphiferous centres of young colonies, 1 week old at 25° C., were cut out with a sterile No. 3 cork borer (6 mm.); following upon this operation, a new ring of rhizomorph initials was produced at a radius of 7–10 mm. from the original colony centre. It so happened that a series of plates, comprising a dextrose-peptone experiment with 20 factorial combinations in triplicate, was immediately available for a repetition of this preliminary experiment; the three replicate plates of each series were treated in the following different ways. On the first plate the rhizomorphiferous centre of the young colony was cut out 1 week after inoculation, with a sterile No. 3 borer, as before; on the second plate the centre was cut round but left in place; on the third plate the young colony was left completely untouched.

The results were similar in all 20 series of dextrose/peptone combinations. In all plates from which the original rhizomorphiferous colony centres had been removed, a new ring of rhizomorph initials arose at a radial distance of 4–6 mm. from the original colony centre. No new rhizomorph initials had arisen either in the colonies in which the centres had been cut round but left in place (so that the original rhizomorphs grew on), or in the undisturbed colonies; it thus appeared that the new rhizomorph initials observed after complete removal of the original rhizomorphiferous colony centre had not developed in response to any kind of wound stimulus. From the results of this experiment it can be concluded that developing rhizomorphs in some way inhibit the formation of fresh initials, either within the original unorganized mycelium growing out first of all from the inoculum disc or from their own fringing mycelium.

Experiment 2. In this experiment the original inoculum discs were left undisturbed but, immediately after inoculation of the plates, a complete isolation ring, 1·5 mm. wide and 15 mm. internal diameter, was cut out of the agar round the inoculum disc; the effect was to give a very small 'agar plate' of 15 mm. diameter. The experiment was carried out on 2 per cent. malt extract+0·15 per cent. peptone agar, and 26 plates were inoculated with 4 mm. marginal discs of mycelium growing on the same medium; 5 plates were left as controls without isolation rings, whilst complete isolation rings were cut round the inoculum discs on the remaining 21 plates, by the use of Nos. 10 and 12 borers, and a sterile needle for picking out the agar in 4 segments.

On the untouched control plates, rhizomorph initials were visible after 7 days, and outwards growth of the young rhizomorphs continued unchecked. After 12 days rhizomorph initials had formed around the inoculum disc on only 7/21 of the 'isolation ring' plates; after 19 days initials had appeared on

one more plate, making 8/21 in all, and no further initials appeared around the inoculum disc on any plate for the remainder of the observation period (35 days). The number of initials formed within the isolation ring on these 8 plates was abnormally low (mean number = 4), and only one young rhizomorph grew far enough to cross the isolation ring; once across the gap, however, it increased greatly in girth, and grew and branched very vigorously. None of the remaining initials grew very far before ceasing growth.

The unorganized mycelium, however, advanced steadily over the central core of agar on all the plates, and had crossed the isolation trench in 18/21 plates by 19 days after inoculation; 9 days later a ring of new rhizomorph initials had formed on the farther side of the isolation trench in 10/21 plates, and after 16 days 20/21 plates showed such a ring of new initials.

In the interpretation of these results two alternative hypotheses were possible, according to whether the isolation trench was inhibiting the production of new rhizomorph initials by preventing inwards or outwards diffusion of soluble substances. The trench might function by preventing the inwards diffusion of nutrients essential for the building-up of a certain threshold concentration of nutrients in the mycelium, which previous experiments have indicated to be essential for rhizomorph initiation. Alternatively the trench might function by preventing diffusion of a hormone-like substance, produced by the growing rhizomorph and inhibiting the production of new initials in its neighbourhood.

Experiment 3. This experiment was designed to discriminate between the two alternative hypotheses outlined above. The simplest method of deciding the issue was by a suitable choice of inoculum disc. If the hormone theory were correct, then rhizomorph initials should be produced freely by an inoculum disc taken from a colony on plain water agar, on which no rhizomorphs are produced (if the inoculum disc used to inoculate the plain water agar be itself low in nutrient status). If the nutrient deficiency theory were correct, on the other hand, then no rhizomorph initials would be produced by such an inoculum disc from a plain water agar colony, but they would be produced by an inoculum disc of sufficiently high nutrient status. The only nutrient likely to be deficient on the agar so far employed in expts. 2 and 3, viz. 2 per cent. malt extract+0.15 per cent. peptone, was nitrogen; the total nitrogen content was *c.* 0·037 per cent. (0·02 per cent. contributed by the peptone and 0·017 per cent. by the malt extract). This concentration was well below the optimum of 0·08 per cent. nitrogen or above, indicated in Table IV. Accordingly, 10 plates of 2 per cent. malt extract+0·15 per cent. peptone agar were inoculated with 4 mm. marginal discs from a colony on an agar containing 2 per cent. dextrose+1·2 per cent. peptone (giving a total of 0·16 per cent. N in the medium)+mineral salts+thiamin (as used for expt. 2 of sub-section 1). Another 10 plates were inoculated with 4 mm. marginal discs from a colony on plain water agar. Immediately after inoculation a complete isolation ring was cut out of the agar around the inoculum disc on each plate, in the same way as for expt. 3.

After 8 days numerous initials had developed around the dextrose-peptone agar inoculum discs on all 10 plates; no initials at all had appeared around the inoculum discs of plain water agar, and none were found later, though observations were continued for 32 days. On three plates similar inoculum discs of mycelium on plain water agar had been placed *outside* the isolation trench as a control; around these inoculum discs rhizomorphs developed normally and abundantly. A further observation worth noting is that although the *number* of initials formed around the dextrose-peptone discs was not affected by the isolation ring (by comparison with similar control discs placed *outside* the ring), yet the subsequent development of these initials was retarded, and none grew far enough to cross the isolation ring; early growth would doubtless have exhausted the additional nitrogen supplied by the inoculum disc.

The results of these experiments show that when a young colony of *A. mellea* starts growth on an agar medium deficient in one or more essential nutrients, the deficiency is made good by inwards diffusion of nutrients from outlying regions of the agar plate; if such diffusion is prevented by means of an isolation trench cut in the agar, then both formation of rhizomorph initials and their subsequent development are depressed, or even inhibited altogether. An obvious corollary of this conclusion is that a growing rhizomorph, together with its branches, is likely so to deplete the surrounding agar of diffusible nutrients that the inception of new rhizomorph initials within the fringing mycelium is rendered impossible. This conclusion is further tested in the following experiment.

Experiment 4. This experiment was designed to test the hypothesis that the rhizomorph with its fringing mycelium is physiologically analogous, in some respects at least, to the root of a higher plant with its root-hairs. The rhizomorph, on this hypothesis, is thought to act as a sink or reservoir for nutrients absorbed and passed on by the fringing mycelium. When the central rhizomorph initials are cut out from a young colony, as in expt. 1, new foci for accumulation of nutrients arise in the unorganized mycelium left behind, and these are the sites at which new rhizomorph initials are formed. This general hypothesis was tested by severing the connexion between the outlying unorganized mycelium growing out from a rhizomorphiferous colony and the remainder of the colony, as follows. After pouring 28 plates with 2 per cent. malt extract+0·15 per cent. peptone agar and allowing the agar to set, a diametrical trench some 2 mm. wide was cut out of the agar with a sterile scalpel, so as to bisect each plate. A 4 mm. marginal inoculum disc of mycelium on the same nutrient agar was placed some 1–2 mm. from the trench on one half only of each split plate; this placing of the inoculum disc had the desired effect of ensuring that no rhizomorph initials were formed on that side of the inoculum disc immediately opposite to the trench, and so no rhizomorphs grew in the direction of the trench. Unorganized mycelium growing out from the young colonies, however, crossed the trench on all plates, and commenced to develop on the other side into a semicircular 'daughter' colony. After 19 days following inoculation the connecting

mycelium between the parent and daughter colonies was severed on 15 plates, and the originally inoculated half of each plate, on which the rhizomorphs had grown almost to the margin of the plate, was removed to prevent re-establishment of a mycelial connexion; the remaining 13 plates were left untouched as controls. Observations on the initiation of rhizomorphs in the daughter colonies were recorded 15 days after severing of the mycelial connexion (Table VII). No further changes were noted in observations made after 22 and 30 days.

TABLE VII

Effect of Mycelial Connexion upon Initiation of Rhizomorphs in Daughter Colonies

	No. of daughter colonies developing rhizomorphs.	Mean No. rhizomorphs in colonies with rhizomorphs.*	Mean No. rhizomorphs, all colonies.†
Mycelial connexion severed	14/15 = 93%	15±3·50	14±3·41
Mycelial connexion left .	5/13 = 38%	6±2·06	2±1·04

* Difference significant ($P = 0{\cdot}05$).
† Difference significant ($P < 0{\cdot}001$).

It is obvious from these results that a severing of the mycelial connexion between the originally inoculated half of a split plate, on which a vigorous parent colony of rhizomorphs had developed, and the daughter colony of mycelium on the other half of the plate, has promoted rhizomorph initiation in the daughter colony. These results thus accord with the hypothesis that the flow of nutrients between a rhizomorph and its fringing mycelium is mainly in the direction of the rhizomorph, and that it is this translocation into the parent rhizomorph which prevents that accumulation of nutrients in the fringing mycelium which would be essential for the formation of new rhizomorph initials. Owing to the widespread occurrence of mycelial anastomoses, which are now recognized to be much more general than was formerly supposed, it is reasonable to assume that all the mycelium of the daughter colony is interconnected by means of such anastomoses with the similarly interconnected mycelium of the rhizomorphiferous parent colony. If this assumption be correct, then it must follow that all the mycelium of the daughter colony will be subjected, to a greater or less extent, to the 'translocation pull' exerted by the rhizomorphs of the parent colony, so long as the mycelial connexion between the two colonies remains intact. The opportunity for production of new rhizomorph initials in the daughter colony will therefore be inversely related to the efficiency of the various mycelial interconnexions here postulated; if such connexions are not completely adequate for translocation of nutrients back to the rhizomorphs of the parent colony, then accumulation of nutrients in the mycelium of the daughter colony will occur, with its necessary consequence in the production of new rhizomorph initials. The actual results of this experiment show that the maintenance of the mycelial connexion between the two colonies has restricted the production of

new initials to approximately two out of five daughter colonies, and that the number of rhizomorphs produced by such a daughter colony is less than half the number produced by a daughter colony rendered independent of the parent by severing the mycelial connexion.

In Pl. II, Fig. 3, is illustrated a typical pair of split plates from this experiment. On the plate from which the original parent colony with rhizomorphs has been removed, the daughter colony on the other half of the plate has formed rhizomorphs; on the plate in which the connexion with the parent colony has been maintained, the daughter colony is one of unorganized mycelium alone.

V. Summary and Conclusions

The isolate of *Armillaria mellea* employed throughout these experiments was unable to utilize nitrate nitrogen; ammonium tartrate was found to be a suitable nitrogen source, though not so good as peptone. Thiamin was essential for growth on a synthetic medium.

When a 4 mm. disc of mycelium taken from the margin of a colony of *A. mellea* on agar is laid on a fresh agar substrate, within 24 hours at 25° C., an outward growth of unorganized mycelium can be seen; rhizomorph initials, which are formed in a circle around the margin of the inoculum disc, can be observed after 7 days. The young rhizomorphs, which start growth later than the unorganized mycelium, have a maximal growth rate some 5 times as great as that of the individual hyphae comprising the mycelium, and hence quickly overtake the mycelial margin of the colony. From the rhizomorphs and their branches, fringing mycelium grows out into the agar, and this imparts a deeply lobed appearance to the whole colony.

An interaction was found between carbohydrate and nitrogen in their effects upon both initiation and subsequent growth of rhizomorphs; as the carbohydrate concentration of the medium was increased, so the optimum concentration of nitrogen for initiation and subsequent growth of rhizomorphs increased with it. At lower levels of carbohydrate (0·125–0·25 per cent. dextrose) the depressing effect of supra-optimal levels of peptone nitrogen was expressed by a reduction in the number of initials produced; at higher carbohydrate levels (1–2 per cent. dextrose) the depressing effect upon subsequent growth of rhizomorphs was greater than that upon initiation, and this has been confirmed by reference to the previous results of Hamada (1940).

On an agar medium of low nutrient status more rhizomorph initials were produced than were able to develop; this was attributed, in part, to a depletion of nutrients by the prior growth of unorganized mycelium.

Under conditions permitting normal development of rhizomorphs on the agar plate, i.e. on a medium of suitable nutrient composition, no independent rhizomorph initials have been observed apart from those originally arising around the inoculum disc. In an attempt to elucidate the factors controlling inception of new initials, the normal course of colony development was mechanically disturbed in various ways, with results that can be interpreted

as follows. A certain threshold nutrient status of the substrate is essential for rhizomorph initiation; once a rhizomorph has started growth upon an agar plate, the inception of independent initials in its neighbourhood is inhibited by the translocation into the rhizomorph of nutrients absorbed by the fringing mycelium, beyond which lies a zone of agar progressively depleted of nutrients as a result of diffusion.

Acknowledgements

I am much indebted to the late Professor F. T. Brooks, C.B.E., F.R.S., for suggestions concerning the manuscript; my thanks are also due to Mr. F. T. N. Elborn, for taking the photographs.

Literature Cited

Benton, V. L., and Ehrlich, J., 1941: Variation in Culture of Several Isolates of *Armillaria mellea* from Western White Pine. Phytopathology, xxxi. 803–11.

Brian, P. W., Curtis, P. J., and Hemming, H. G., 1947: Glutinosin: a Fungistatic Metabolic Product of the Mould *Metarrhizium glutinosum* S. Pope. Proc. Roy. Soc., B, cxxxv. 106–32.

Fisher, R. A., and Yates, F., 1949: Statistical Tables. Oliver and Boyd, Edinburgh.

Garrett, S. D., 1946: A Multiple-point Inoculating Needle for Agar Plates. Trans. Brit. Mycol. Soc., xxix. 171–2.

—— 1949: A Study of Violet Root Rot. II. Effect of Substratum on Survival of *Helicobasidium purpureum* Colonies in the Soil. Ibid., xxxii. 217–23.

—— 1950: Ecology of the Root Inhabiting Fungi. Biol. Rev., xxv. 220–54.

—— 1951: Ecological Groups of Soil Fungi: a Survey of Substrate Relationships. New Phytol., l. 149–66.

Hamada, M., 1940: Physiologisch-morphologische Studien über *Armillaria mellea* (Vahl) Quél., mit besonderer Rücksicht auf die Oxalsäurebildung. Jap. J. Bot., x. 387–464.

Hawker, L. E., 1950: Physiology of Fungi. University of London Press.

Reitsma, J., 1932: Studien über *Armillaria mellea* (Vahl) Quél. Phytopath. Z., iv. 461–522.

EXPLANATION OF PLATE

Illustrating S. D. Garrett's article on 'Rhizomorph Behaviour in *Armillaria mellea* (Vahl) Quél. I. Factors controlling Rhizomorph Initiation by *A. mellea* in Pure Culture'.

Fig. 1. A 10-day-old colony of *Armillaria mellea* growing on agar containing 2 per cent. dextrose + 0·6 per cent. peptone + mineral salts + thiamin. Note circle of young rhizomorphs, which have arisen round margin of 4 mm. inoculum disc. Their growing tips have not yet reached the mycelial margin of the colony.

Fig. 2. The same colony 13 days later. The rhizomorphs have overtaken in growth the original mycelial margin of the colony. From these growing rhizomorphs and their branches, fringing mycelium has grown out, imparting a characteristically lobed appearance to the colony.

Fig. 3. Parent and daughter colonies of *A. mellea* growing on 'split plates'. For explanation, see text, p. 78.

FIG. 1

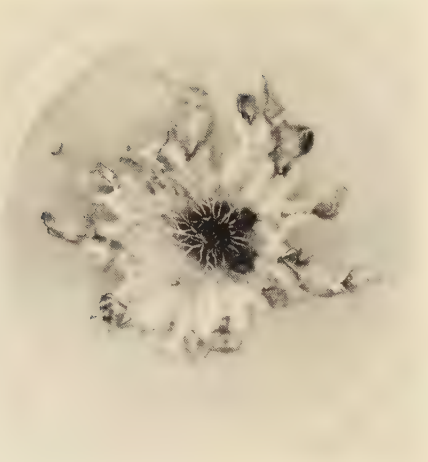

FIG. 2

FIG. 3

S. D. GARRETT

The Control of Inter-pollination between Sweet and Flint Corn

BY

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With two Figures in the Text

Abstract

The breeding system of sweet corn, a sugary seeded race of *Zea mays* L., was studied by randomly planting equal numbers of sweet and flint plants and determining the amount of cross-pollination measured by the contamination on sweet ears. It was found that inter-crossing with flint varied significantly from plant to plant; the out-crossing generally was lower than anticipated. The effects of date of silking and tasselling, and protandry of sweet plants on their out-crossing was demonstrated by using multiple regression analysis. Even so, as prediction was still not satisfactory other factors must have been involved. These variables were all highly correlated. A relation between contamination and temporal isolation was established; this was similar to that for spatial isolation. It was postulated that there may have been genetical control of inter-pollination, as later ears on the same sweet-corn plants were not less contaminated than earlier ones and because out-crossing was generally low throughout the experiment.

Introduction

A PRIMARY difference between sweet corns and forms of starchy maize lies in the endosperm nature of the caryopsis. This relationship is governed by the alleles *Su* and *su*. The sugary-starchy condition is useful in studying the breeding system of maize because the two types of caryopses can be differentiated while they are still on the ear.

Jones (1928) questioned the early observations of Gartner, who had noticed that two varieties of maize, a yellow-seeded dwarf and a tall red-seeded type, did not cross spontaneously; usually even most diverse types of maize will readily cross. Differences in flowering times could have accounted for this. Even so, little is known to what extent not only different varieties but also plants within a maize crop naturally intercross.

An investigation was therefore made in an attempt to clarify the situation by determining the amount of natural cross-pollination between sweet corn and flint maize when the former was given free choice of pollen under field conditions. Many of the earlier sweet corns are suspected as being derivatives of flint corns; therefore both sweet- and flint-corn plants should be completely inter-fertile at least when hand pollinated (East, 1909).

[Annals of Botany, N.S. Vol. XVII, No. 65, January, 1953]

Experimental Methods

Ninety-eight plants each of a flinty and sugary type were sown at random on May 24, 1945, in a square block, each plant being 2 ft. from its neighbour.

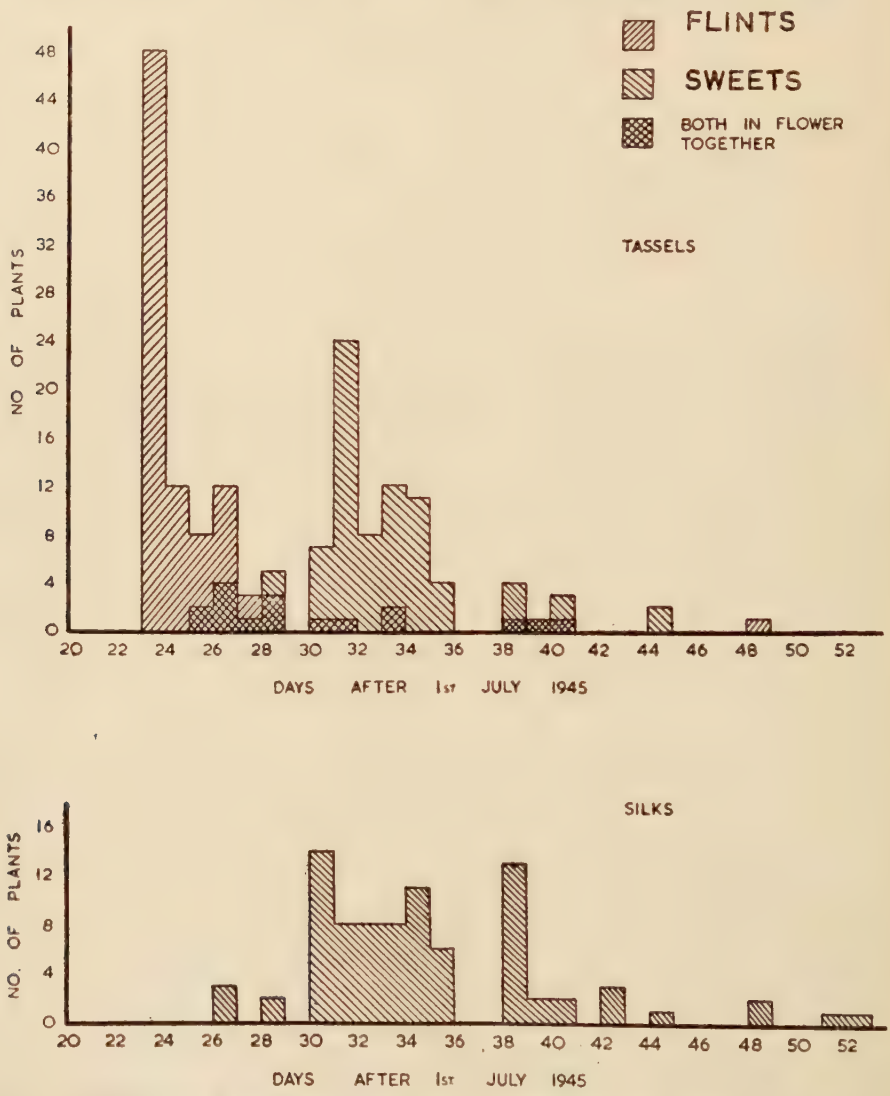

Fig. 1. Distribution of tasselling of both flint and sweet corns, and silking times of sweet corn.

The block was divided into two plots for randomization so that equal numbers of both types occurred in eastern and western plots. Three flinty and eleven sweet plants failed to reach the flowering stage. Records were made of the dates when anthers and silks first appeared.

Fig. 1 illustrates the frequency for both sweet and flint plants daily coming into tassel for the first time and also the daily frequency of sweet plants which silked for the first time. A maize plant actually continues to shed pollen for several days after the first anthers appear. All measurements were taken from days from July 1, 1945. The protandry was also calculated by taking the difference between silking and tasselling date of individual plants. When the caryopses were beginning to ripen in the autumn it was possible to distinguish between the two types of seed that had set on ears of the sweet-corn plants.

Records were made of sugary and starchy seeds on one, two, or occasionally three ears which ripened on each sweet-corn plant. The percentage of starchy seed on each ear was calculated and used as an estimate of inter-pollination. Angular transformations and the mean angle per plant were determined to reduce error during analysis of variance calculations (see Appendix).

Analysis of the Results

Inter-plant variation

To determine whether the amount of contamination varies from plant to plant, an analysis of variance was made on the original data converted from percentages to angles, there being 84 plants with 158 ears. It was found (Table I) that probability P was less than 0·001, so that there were genuine differences between the various plants of the sweet-corn variety in their capacity to be pollinated by flint-corn pollen.

Table I

Analysis of Variance of the Original Data in Percentages converted to Angles

	S.S.	N.	M.S.	V.R.	P.
Between plants . .	7,749·19	83	93·36	2·08	< 0·001
Within plants (Error) .	3,315·24	74	44·80		
Total . . .	11,064·43	157			

For the purposes of discussion the mean angle may be taken to represent the actual amount of fertilization of the sweet-corn ovaries by flint-corn pollen; the theoretically expected inter-pollination is 50 per cent. (45°) provided that the 'sweet' plants have free choice of equal amounts of both types of pollen when their silks are receptive. But factors such as selective pollen competition and differences in dates of pollen-shedding by individual 'flint' and 'sweet' plants could modify the proportion of the two types of caryopses on the sweet-corn ears. The mean percentage of flint caryopses on sweet-corn ears actually was 1·85 per cent. (7·8°), giving a value of $\chi^2 = 195{\cdot}26$, which is highly significant. Thus it is inferred that the out-crossing with flint pollen is very much lower than would be expected if conditions were equal for each pollen type.

It therefore appears that given the choice of sweet and flint pollen in the above population, sweet pollen has been more effective. Now the chances of

receiving only sweet-corn pollen should increase the later the sweet-corn plants flowered relative to the flint-corn plants. Temporal relations must, therefore, be taken into account.

Influence of silking time (x_1), *tasselling time* (x_2), *and protandry* (x_3)

A multiple regression analysis is necessary to determine the relative effectiveness of these variables, suspected of influencing inter-breeding activity. The statistical procedure is given in the Appendix. However, in the demonstrated example for x_1 and x_2, contamination is not statistically dependent on silking or tasselling times. But regression, or b, coefficients can also be calculated from x_1 with x_3, and x_2 with x_3. These values are given in Table II. The symmetry of the b coefficients may be noted: b_1 and b_2 are always negative. This implies that contamination tends to decrease the later sweet-corn plants silk, and the later the time of their pollen shedding is in relation to that of flint-corn plants, most of which were earlier (Fig. 1.) Regression constant b_3 is positive or negative according to whether x_2 or x_1 was omitted.

TABLE II

b Coefficients for x_1, x, *and* x_3

Variables used.	$b_{1.}$	$b_{2.}$	$b_{3.}$	S.S. due to b.	S.S. due to regression y.	Total.	Significance.
x_1 and x_2	−0·33515	−0·65815	—	1,301·3970	3,676·9736	4,978·3706	Highly significant.
x_1 and x_3	−0·99338*	—	+0·65815	1,301·5387	3,676·8319	4,978·3706	
x_1 and x_3	—	−0·99330*	−0·33515	1,301·3985	3,676·9721	4,978·3706	
x_3	−0·70268*	—	—	1,173·0211	3,805·3495	4,978·3706	Highly significant.
x_2	—	−1·08682*	—	1,225·1518	3,753·2188	4,978·3706	Highly significant.
x_3	—	—	−0·71364	386·8312	4,591·5394	4,978·3706	0·01–0·001

* Highly significant.

Table II also shows the sums of squares due to the regression constants when only one variate was used. It is seen that those of x_1 and x_2 are close, but the sum of squares for x_3 alone is low. For single variates all three constants are negative. The negative regression of contamination with silking time is to be expected with flint-corn plants tasselling before the sweet-corn: plants silking later are less likely to obtain flinty pollen. The negative regression for tasselling is also to be expected as proportionally more sweet than flinty pollen would be shedding at later periods. Taken by itself the regression constant for protandry is negative, indicating that with greater protandry there is less likelihood of the silks receiving flint pollen. This is a reflection of the greater chance of inbreeding should silks appear while, on the same plant, tassels above them are dropping pollen. Conversely, increased protogyny means increased outbreeding.

Tests of significance were made on all regression coefficients: b_1 and b_2 were significant when calculated by omitting x_2 or x_1, but not when x_1 and x_2 were used together. All regression coefficients taken singly were highly significant.

Prediction Formula

Using silking times (x_1) and tasselling times (x_2), the predicted contamination, Y, is given by Mather (1946) as

$$\begin{aligned} Y &= \bar{y}+b_1(x_1-\bar{x}_1)+b_2(x_2-\bar{x}_2) \\ &= 7{\cdot}7995-0{\cdot}3351(x_1-34{\cdot}1667)-0{\cdot}6582(x_2-32{\cdot}0952) \\ &= 40{\cdot}3738-0{\cdot}3351x_1-0{\cdot}6582x_2. \end{aligned}$$

To determine how close the predicted values are to actual values, substitution was made in the first ten lines of the original data. These values are given in Table III. It is clear that prediction is not as good as might be hoped. Other factors besides silking and tasselling must play a role in affecting contamination.

Table III

Actual (y) and Predicted (Y) Values of Contamination (in angles) in Ten Plants using Silking and Tasselling Times for Prediction

Plant No.	y	Y	$y-Y$
A 3	9·12	8·60	+0·52
5	7·02	5·25	+1·77
7	6·32	9·25	−2·93
9	34·80	14·88	+19·92
11	3·57	5·92	−2·35
12	6·77	5·26	+1·51
13	3·86	8·58	−4·72
14	2·17	7·26	−5·09
B 2	10·30	7·93	+2·37
4	0·00	0·98	−0·98

Correlation between silking and tasselling

It is already known that, regardless of sowing date, mean tasselling and silking dates are good indicators of when the crop will be ready for picking (Singleton, 1948; Haskell, 1950). It is therefore of interest to determine what kind of correlation there is between silking and tasselling of sweet-corn plants in England. Correlation coefficients can be determined from the present data using

$$r_{x_1 x_2} = \frac{S[(x_1-\bar{x}_1)(x_2-\bar{x}_2)]}{[S(x_1-\bar{x}_1)^2 . S(x_2-\bar{x}_2)^2]}.$$

For x_1 and x_2, $r = +0{\cdot}85$, which is very high. The regression of x_1 on x_2 is given by $b_1 = \frac{1{,}326{\cdot}667}{1{,}037{\cdot}238} = 1{\cdot}279$, while the regression of x_2 on x_1 is $b_2 = \frac{1{,}326{\cdot}667}{2{,}375{\cdot}667}$ $= 0{\cdot}558$. The correlation coefficients and their regressions for x_1, x_2, and x_3 are given in Table IV. It is clear that protandry and silking times are highly correlated in sweet corn. This is important in this experiment, as the earliness or lateness of a plant within a variety tends to influence the proportion of the two kinds of pollen reaching the sweet styles.

TABLE IV

Correlation and Regressions between Silking Times (x_1), Tasselling Times (x_2), and Protandry (x_3) in a Sweet-corn Variety

Using	Correlation coefficient r.	b_1	b_2	b_3
x_1 and x_2	+0·85	1·28	0·56	—
x_1 and x_3	+0·78	1·38	—	0·44
x_2 and x_3	+0·33	—	0·38	0·28

Contamination and temporal isolation

In order to determine more clearly the relations of the three variates with contamination, the data were rearranged so that the values for plants coming into silk or tassel on a particular day were pooled and the mean contamination (in angles) determined. The data in percentages are given in Fig. 2. The graphs for both x_1 and x_2 are very similar, the fall in contamination from the time of first flowering being extraordinarily rapid. There was almost no contamination by the tenth day.

The graphs in Fig. 2 may be compared with those of Bateman (1947) and Griffiths (1951) who were relating contamination with spatial isolation, not temporal. The shapes of the graphs are remarkably similar. In Fig. 2, inset, a graph is given of mean contamination plotted against protandry in days. It is not easy to see how protogyny affects contamination, but it is quite clear that contamination falls off the greater the protandry, which in maize is not absolute. No doubt the decrease is largely due to the relatively high correlation between protandry and silking-tasselling times.

Effect of ear position and ear number per plant

The contamination of ears in order of their appearance on a particular day were compared. Normally the first ears are those highest on the stems. Plants were also classified according to the number of fertile ears they bore. These data are given in Table V.

TABLE V

Mean Percentage Contamination on Different Ears of Plants bearing Various Number of Ears

Plants bearing	Total No.	Ear Position				Mean.
		1st.	2nd.	3rd.	4th.	
1 ear	34	4·21	—	—	—	4·21
2 ears	30	2·01	5·57	—	—	3·79
3 ears	17	2·77	3·28	2·72	—	2·92
4 ears	3	2·11	2·36	5·21	2·83	3·12
	84					
Mean		2·78	3·74	3·97	2·83	

There is no indication that on the average later ears have less contamination than earlier ears on the same plants. It was expected that they would have less because there should be comparatively less flint-corn pollen prevailing. Position of the ears on the plants was, therefore, not a factor influencing contamination in this experiment. Thus it is deduced that although there was sufficient flint-corn pollen to allow for a much greater contamination in the earlier ears, which were produced considerably before the second ones, yet

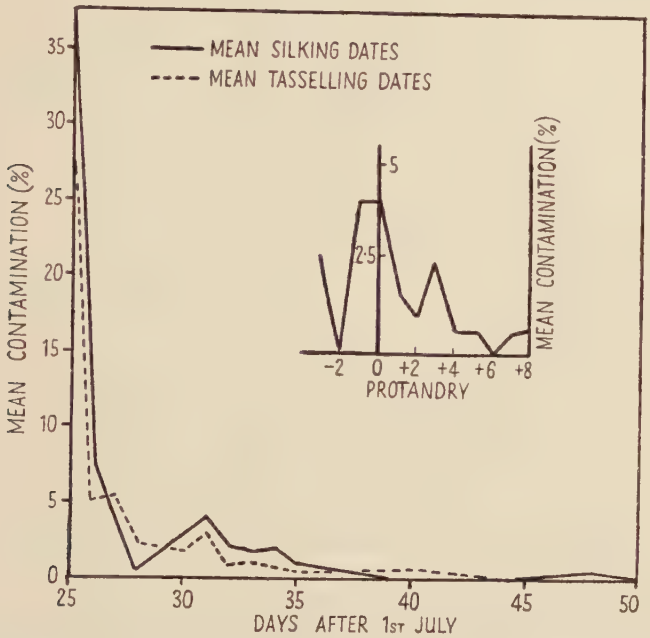

FIG. 2. Percentage contamination with flint seeds on sweet-corn ears plotted against the flowering times of sweet-corn plants, to show the relation between cross-pollination of sweet corn by flint corn and temporal isolation. Inset: Relation between percentage contamination with flint corn and protandry in sweet corn.

a greater amount of contamination did not occur. Some factor was therefore reducing cross-pollination throughout the experiment.

Thus whichever way the data are examined, an indication is left that there was at least another factor which determined the extremely low contamination. This is most likely to be genetical.

DISCUSSION

Maize is a wind-pollinated crop with preponderance of natural cross-pollination. Although there is much chance of contamination by crossing, it is not a complete out-crosser like rye-grass and cocksfoot (Bateman, 1949). When calculating the deviation from expected it was assumed that there could be 100 per cent. outcrossing with equal choice of sweet- or flint-corn pollen. The actual difference from 100 per cent. is sufficiently small not to introduce

too large an error. The amount of flinty seeds on sweet-corn plants was considerably lower than anticipated; this could only partly be attributed to most flinty pollen shedding before the sweet-corn styles were ready, as indicated by Fig. 1.

Kemp and Rothgeb (1943) found in Maryland maize populations approximately 96 per cent. out-crossing on any plant under their field conditions, the amount tending to vary with weather conditions at flowering time. Kiesselbach (1922) also found less than 0·7 per cent. selfing in a field of white dent maize interplated with yellow dent having a similar flowering period. This strongly suggests that any excess of sugary seeds in my experiments is unlikely to result from selfing by individual plants. Table I shows that they significantly differ in receptivity to flint pollen, and Lonnquist and Jugenheimer (1943) have already ascertained different cross-potency of pollen from various lines with silks of a seed parent. From the present experiment it is not easy to deduce how much individual plants are self-pollinated.

Kiesselbach (1922) in Nebraska found that pollen fertilizing dent maize falls on silks for 3 days at the time of maximum pollen-shedding by the same plant. An average plant in an average year sheds most of its pollen about the time when its ear is pollinated. There is thus ample opportunity for self-fertilization. This has economic interest as selfed maize seeds yield only one-third that of out-crossed. However, the overwhelming preponderance from other plants of pollen grains randomly distributed in the air around a maize field reduces selfing possibilities. Jones and Newell (1946) in Nebraska found most pollen was shed on the fourth day of tasselling, gradually ceasing by the twelfth. If this is similar under English climatic conditions there should have been sufficient flint pollen competing with the sugary even though most flints tasselled 8 days earlier than most sweet corns. In Nebraska most pollen is shed between 9 and 10 a.m., and although this may differ somewhat in England, breeding experience indicates that most sweet-corn pollen is available in the mornings and little in afternoons. Dispersal of maize pollen is known to go with prevailing wind direction (Bateman, 1947; Jones and Brooks, 1950).

As the plants were 2 ft. apart they were sufficiently close for complete pollination, for detasselled plants 4 ft. from a pollen source give a relatively full seed-set (Haskell and Dow, 1951). Bateman (1950) found that pollen dispersal by wind is leptokurtic, with higher proportions at short range, so perhaps relative positions of flint plants to sweets influenced contamination. But on the average this cannot be very important, as although each plant, except for border ones, was 2 ft. or $\sqrt{(2^2+2^2)} = 2{\cdot}8$ ft. from its neighbour, both varieties were planted at random. However, nearest plants of *Lolium perenne* make the largest pollen contribution to seed-setting in this species (Griffiths, 1951).

Ideally flints should have had the same distribution of flowering as sweet corns, but this situation is difficult to attain. Hence multiple regressions must be used to estimate the relative importance of the three variates, viz. silking

and tasselling times and protandry of sugary plants, in relation to contamination. Multiple regressions have already been useful for measuring joint relationships in maize, e.g. those of temperature and precipitation on yields (Hendriks and Scholl, 1943).

One of the three variates is redundant as the regression from any two gives the same sum of squares (S.S.). The S.S. due to tasselling alone is not much less, nor is that for silking much less than that of their joint regression. On the other hand, the S.S. for protandry alone is low. Thus actual time of flowering, especially pollen shedding, is considerably more important in influencing contamination than the degree of protandry, though all are linked together. In contamination of sweet corn by flint, Bateman (1947) emphasized the importance of variation in contamination due to isolation distance, with protandry being important where plants were sparsely spaced. Sequence of silking also produced variation on ears of the same plant, earlier ears tending to have more contamination than later ones. Yet the present data in Table V do not show this. The difference could, however, be masked by the generally low contamination throughout the experiment. This may also account for the poor prediction given by the regressions (Table III).

The importance of temporal isolation is clear from the regressions and graphs. It is of the same general nature as spatial isolation (Fig. 2). Where flowering of varieties or inbreds for seed do not overlap they need not be spatially isolated as temporal isolation is sufficient. As maize pollen lasts only 24 hours there is no danger from old pollen. Because of the high correlation between silking and tasselling in sweet corn, both are not necessary for predicting time of picking (cf. Haskell, 1950).

As the variables only partially account for variation in contamination within the crop, the possibility may be considered of a genetical influence reducing cross-pollination with flint. Genetic barriers to crossability in maize and its relatives are known: Mangelsdorf and Reeves (1931) found some varieties crossed more easily with *Tripsacum* than others.

Mangelsdorf and Jones (1926), comparing effects of mixed pollinations of white popcorn and yellow sweet-corn pollen on both types of styles, found selective action which was due to differences in pollen-tube growth; sweet-corn plants only slightly preferred sweet-corn pollen. This is different from the inter-pollination barrier between *Antirrhinum majus* and *A. glutinosum*, where pollen mixtures are equally effective either way (Mather, 1947), the barrier being flower discrimination by bees which keep to one species. Style length could be another factor in maize, but might be more important in species-hybrids than when pollen grains differing by a single gene are competing. Segregating crosses between starchy and sweet show less than the expected 25 per cent. sweets, and this has been attributed to *Su* pollen being able to grow better in competition; but Jones (1924) demonstrated that pollen carrying the dominant factor only accomplishes fertilization better than pollen with the recessive in a sporophyte carrying the dominant factor. Pollen-grain differences alone are not sufficient to produce fertilization differences.

There is a gamete gene (*Ga*) in maize which can cause selective fertilization and reduction in contamination of sweet corn by field maize (Perry, 1945). For instance, Emerson (1934) found the percentage fertilized by pollen mixtures gave an average of 50 per cent. on *ga* plants and an average of 4·0 per cent. on *Ga* plants. He concluded that the percentage of functioning pollen in competition with *Ga* pollen on *Ga* silks was from 0 to 2·5 per cent. This value agrees reasonably well with 2·0 per cent. out-crossing to flint obtained in the present experiment. Although there is here no direct genetical evidence for this, the *Ga* gene might have been carried by sweet-corn pollen and *ga* by flint. It might be expected to give four times as much sweet as flint if equal amounts of pollen were present under equal conditions (Mangelsdorf and Jones, 1926). Such a genetical barrier to inter-pollination is important where both sweet and starchy corn have to be grown together, as in some parts of the U.S.A. It might also explain how American Indians have maintained their stocks of sweet corn so successfully even though carrying the recessive gene.

Summary

The inter-pollination of sweet corn (*su*) with flinty maize (*Su*) was studied. Ninety-eight plants of each were grown at random 2 ft. apart in a block. Silking and tasselling times were recorded. Flints shed pollen somewhat earlier than sweets. Ripe ears on sweet plants were scored for sugary and starchy seeds. Percentages of contamination were calculated and transformed to angles for analysis.

There were significant differences between plants in their capacity to be pollinated by flint pollen; contamination was considerably lower than expected. Multiple regression analyses were made to show how silking date, tasselling date, and protandry of sweet plants affected contamination. A prediction formula was obtained from these, but was not satisfactory.

These variables had strong positive correlations. There was also a relation between contamination and temporal isolation; contamination falling off rapidly with later flowering. Neither ear position nor number of ears per plant affected contamination.

The general low contamination may have been due to a genetical factor in the sweet-corn stock which aided sugary pollen to compete more successfully than flint pollen on sugary styles.

Appendix

Angular Transformation of Percentages

Angular transformations, called 'angles', are due to C. I. Bliss. They are now frequently used instead of actual percentages, especially for calculations involving the analysis of variance. A conversion table is available in Mather (1946), who discusses the theory and applications of transformed percentage data.

Multiple Regression Analysis

To investigate the influence of three variables, silking time, tasselling time, and protandry, let

y = average angle of contamination, transformed from percentage of flint seeds setting on sweet-corn ears;

x_1 = silking time from July 1 of sweet-corn plants;

x_2 = tasselling time from July 1 of sweet-corn plants;

$x_3 = x_2 - x_1$ = protandry of sweet-corn plants.

Then the formula given by Mather (1946) for calculating the multiple regressions of y on the three variates x_1, x_2, and x_3 are:

$$b_1 S(x_1-\bar{x}_1)^2+b_2 S[(x_1-\bar{x}_1)(x_2-\bar{x}_2)]+b_3 S[(x_1-\bar{x}_1)(x_3-\bar{x}_3)] = S[y(x_1-\bar{x}_1)]$$
$$b_1 S[(x_1-\bar{x}_1)(x_2-\bar{x}_2)]+b_2 S(x_2-\bar{x}_2)^2+b_3 S[(x_2-\bar{x}_2)(x_3-\bar{x}_3)] = S[y(x_2-\bar{x}_2)]$$
$$b_1 S[(x_1-\bar{x}_1)(x_3-\bar{x}_3)]+b_2 S[(x_2-\bar{x}_2)(x_3-\bar{x}_3)]+b_3 S(x_3-\bar{x}_3)^2 = S[y(x_3-\bar{x}_3)],$$

where b_1, b_2, and b_3 are constants.

Appendix Table I

Values for calculating Multiple Regressions

n	84		
$S x_1$	2,870	$S(x_1-\bar{x}_1)^2$	+2,375·6667
$\bar{x}_1$	34·1667	$S(x_2-\bar{x}_2)^2$	+1,037·2381
$S x_2$	2,696	$S(x_3-\bar{x}_3)^2$	+ 759·5714
$\bar{x}_2$	32·0952	$S[y(x_1-\bar{x}_1)]$	−1,669·3433
$S x_3$	174	$S[y(x_2-\bar{x}_2)]$	−1,127·2862
$\bar{x}_3$	2·0714	$S[y(x_3-\bar{x}_3)]$	− 542·0571
$S y$	655·1667	$S[x_1(x_2-\bar{x}_2)]$	+1,326·6667
$\bar{y}$	7·7995	$S[x_1(x_3-\bar{x}_3)]$	+1,049·0000
		$S[x_2(x_3-\bar{x}_3)]$	+ 289·4286
		$S(y-\bar{y})^2$	+4,978·3706

n = number of sweet-corn plants.
x_1 = silking times from July 1.
x_2 = tasselling times from July 1.
$x_3 = x_2 - x_1$ = protandry.

y = mean angle (transformed from percentages) of flint seeds = contamination.

The right sides are replaced with 1, 0, 0; 0, 1, 0; 0, 0, 1 respectively and substitution is made in these formulae of actual values from Appendix Table I. This gives:

$$2{,}375{\cdot}6667b_1+1{,}326{\cdot}6667b_2+1{,}049{\cdot}0000b_3 = 1, 0, 0 \qquad (\alpha)$$
$$1{,}326{\cdot}6667b_1+1{,}037{\cdot}2381b_2+\ 289{\cdot}4286b_3 = 0, 1, 0 \qquad (\beta)$$
$$1{,}049{\cdot}0000b_1+\ 289{\cdot}4286b_2+\ 759{\cdot}5714b_3 = 0, 0, 1 \qquad (\gamma)$$

When attempting to solve these equations it is found that after first eliminating b_1, b_3 goes out as well when b_2 is eliminated. This is not surprising as inspection shows $\alpha = \beta+\gamma$. This limitation of the multiple regression analysis where x_3 is a difference between x_1 and x_2 will be discussed elsewhere. It is clear then that any two variates are sufficient to describe the relation between flowering times and contamination. Three pairs of equations using x_1 with x_2, x_1 with x_3, and x_2 with x_3 were therefore solved. For instance, using x_1 with x_2 and substituting in the formulae:

$$b_1 S(x_1-\bar{x}_1)^2+b_2 S[(x_1-\bar{x}_1)(x_2-\bar{x}_2)] = 1, 0$$
$$b_1 S[(x_1-\bar{x}_1)(x_2-\bar{x}_2)]+b_2 S(x_2-\bar{x}_2) = 0, 1$$

gives

$$b_1\ 2{,}375{\cdot}6667+b_2\ 1{,}326{\cdot}6667 = 1, 0$$
$$b_1\ 1{,}326{\cdot}6667+b_2\ 1{,}037{\cdot}2381 = 0, 1$$

On solving, the roots of these quadratic equations are placed in the following c-matrix:

c_{11}	c_{12}
+0·0014731651	−0·00188423603
c_{21}	c_{22}
−0·00188423603	+0·00337410728

Then
$$\begin{aligned} b_1 &= c_{11} S[y(x_1-\bar{x}_1)]+c_{21} S[y(x_2-\bar{x}_2)] \\ &= -(0{\cdot}0014731665 1 \times 1{,}669{\cdot}3433)+(0{\cdot}00188423603 \times 1{,}127{\cdot}2862) \\ &= -0{\cdot}335147 37, \end{aligned}$$

and
$$\begin{aligned} b_2 &= c_{12} S[y(x_1-\bar{x}_1)]+c_{22} S[y(x_2-\bar{x}_2)] \\ &= +(0{\cdot}00188423603 \times 1{,}669{\cdot}3433)-(0{\cdot}00337410728 \times 1{,}127{\cdot}2862) \\ &= -0{\cdot}658147874, \end{aligned}$$

also
$$\begin{aligned} S(y-Y)^2 &= S(y-\bar{y})^2-b_1 S[y(x_1-\bar{x}_1)]-b_2 S[y(x_2-\bar{x}_2)] \\ &= 4{,}978{\cdot}3706-(0{\cdot}335147 37 \times 1{,}669{\cdot}3433)-(0{\cdot}658147874 \times 1{,}127{\cdot}2862) \\ &= 3{,}676{\cdot}9736. \end{aligned}$$

Hence the sum of squares due to b is 4,978·3706−3,676·9736 = 1,301·3970.

Then
$$V_y = \frac{S(y-Y)^2}{n-3} = \frac{3{,}676{\cdot}9736}{81} = 45{\cdot}3947,$$
$$S_{b_1} = \sqrt{V_{b_1}} = \sqrt{(c_{11} V_y)} = 0{\cdot}2585,$$

and
$$S_{b_2} = \sqrt{V_{b_2}} = \sqrt{(c_{22} V_y)} = 0{\cdot}3913.$$

Testing for significance we have

$$t_{[81]} = \frac{b_1-0}{s_{b_1}} = \frac{0{\cdot}3351}{0{\cdot}2585} = 1{\cdot}29 \text{ and } P = 0{\cdot}20;$$

$$t_{[81]} = \frac{b_2-0}{s_{b_1}} = \frac{0{\cdot}6581}{0{\cdot}3913} = 1{\cdot}68 \text{ and } P = 0{\cdot}10.$$

Acknowledgements

The writer wishes to thank Professor K. Mather, F.R.S., for providing the experimental data and suggesting use of multiple regressions, and Mr. M. B. Crane, F.R.S., for support of this study.

Literature Cited

Bateman, A. J., 1947: Contamination in Seed Crops. II. Wind Pollination. Heredity, i. 235–46.
—— 1949: The Contamination of Seed. Farming, iii. 106–9, 118.
—— 1950: Is Gene Dispersal Normal? Heredity, iv. 353–63.
East, E. M., 1909: A Note concerning Inheritance in Sweet Corn. Science, xxix. 465–7.
Emerson, R. A., 1934: Relation of the Differential Fertilization Genes Ga*Ga* to certain other Genes of the Su-Tu Linkage Group of Maize. Genetics, xix. 137–56.
Griffiths, D. J., 1951: The Liability of Seed Crops of Perennial Ryegrass (*Lolium perenne*) to Contamination by Windborne Pollen. J. Agric. Sci., xl. 9–38.
Haskell, G., 1950: Studies with Sweet Corn. II. Sowing Dates and Plant Characters. Emp. J. exp. Agric., xviii. 1–7.
—— and Dow, P., 1951: Studies with Sweet Corn. V. Seed-settings with Distances from Pollen Source. Ibid., xix. 45–50.
Hendriks, W. A., and Scholl, J. C., 1943: Techniques in measuring Joint Relationships: the Joint Effects of Temperature and Precipitation on Corn Yields. N. Carolina A.E.S., Tech. Bull. No. 74.
Jones, D. F., 1924: Selective Fertilization among the Gametes from the Same Individual. Proc. Nat. Acad. Sci., x. 218–21.
—— 1928: Selective Fertilization. Chicago.
Jones, M. D., and Brooks, J. S., 1950: Effectiveness of Distance and Border Rows in preventing Outcrossing in Corn. Oklahoma A.E.S., Tech. Bull., T-38.
—— and Newell, L. C., 1946: Pollination Cycles and Pollen Dispersal in Relation to Grass Improvement. Nebraska A.E.S., Bull. No. 148.
Kemp, W. B., and Rothgeb, R. G., 1943: Selection and Genetic Responses in a Segregating Maize Population. Maryland A.E.S., Bull. No. A 26.
Kiesselbach, T. A., 1922: Corn Investigations. Nebraska A.E.S., Bull. No. 20.
Lonnquist, J. H., and Jugenheimer, R. W., 1943: Factors affecting the Success of Pollination in Corn. J. Amer. Soc. Agron., xxxv. 923–33.
Mangelsdorf, P. C., and Jones, D. F., 1926: The Expression of Mendelian Factors in the Gametophyte of Maize. Genetics, xi. 423–55.
—— and Reeves, R. G., 1931: Hybridization of Maize, *Tripsacum* and *Euchlaena*. J. Hered., xxii. 329–43.
Mather, K., 1946: Statistical Analysis in Biology. 2nd edn. London.
—— 1947: Species Crosses in *Antirrhinum*. 1. Genetic Isolation of *A. majus* and *A. glutinosum*. Heredity, i. 175–86.
Perry, H. S., 1945: The Ga Gene as a Means of Reducing Contamination of Sweet Corn. J. Hered., xxxvi. 131–4.
Singleton, W. R., 1948: Hybrid Sweet Corn. Conn. A.E.S., Bull. No. 518.

Studies on the Physiology of Nodule Formation

IV. The Mutual Inhibitory Effects on Nodule Production of Plants grown in Association

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With six Figures in the Text

Abstract

The number of root nodules formed on clover in test-tube culture on agar depends directly upon the volume of the medium and inversely upon the number of seedlings present per unit volume of medium. Plants which form nodules early in seedling development inhibit nodule formation on an associated plant more strongly than plants which normally nodulate later. Among all the other types of plants examined, viz. effectively or ineffectively responding plants, sparsely or abundantly nodulating plants, and resistant or normally susceptible plants, it was shown that the mutual inhibiting effects when grown in pairs are everywhere the same in spite of large differences in plant size. Similar inhibitory effects were found in the presence of non-leguminous plants. Successive replanting of the same agar slope also leads to inhibition of infection.

The nodulation of lucerne and vetch is similarly affected by the presence of a companion plant. Nodulation is always reduced by the presence of a plant of the same species, but among the heterogeneous associations, consisting of members of two different cross-inoculation groups, with either one or both of the appropriate strains of nodule bacteria present, inhibition occurs in some associations and not in others. In such mixed associations inhibition is not related to the numbers of bacteria present or to the pH of medium. The hypothesis is put forward that nodulation may be inhibited by specific substances secreted by the root.

Introduction

THE number of nodules formed on a clover plant grown in a small volume of medium is reduced proportionally by the presence of a second or third plant so that the total number of nodules produced within a container of standard size tends to a constant value irrespective of the number of seedlings present. This number is, moreover, directly related to the volume of the medium available for growth of the roots (Nutman, 1945). These relationships might be attributed to competition between the plants for nutrients, carbon dioxide, or light, leading directly to a limitation of the growth of the individual root systems and thus indirectly to a reduction in the number of nodules formed. Alternatively the interaction between plants may

[Annals of Botany, N.S. Vol. XVII, No. 65, January, 1953.]

be due to exudation from the root of a substance inhibitory to infection, larger amounts of inhibitor accumulating in a culture containing more than one plant, and an inhibitory concentration arising more rapidly in a smaller than in a larger volume of medium.

The object of this paper is to examine the influence of the volume of medium and density of planting on nodulation and in particular to distinguish, if possible, between the effects of competition and inhibition.

Material and Methods

The method of growing nodulated clover plants on slopes of an agar medium in test-tubes has been described in earlier papers of this series. In the first experiments here reported the volume of medium in each tube was not standardized, but in later experiments the initial weight or volume of each culture was accurately controlled and periodically adjusted by adding water or culture solution. In most experiments the volume adjustment was made by using an agar slope of 8 ml. to which 2 ml. of a liquid medium of the same composition was added. A mark was made on the tube at the level of the liquid meniscus and subsequent watering was made to this level to replace the water lost.

Experimental

In the course of experiments designed for other ends, attention was first directed to the inverse relation between the number of nodules per plant and the number of plants present in each culture tube. In these experiments series of test-tube cultures of clover were set up in which each tube was originally planted with two seeds. Some seeds, however, failed to germinate so that a comparison could be made of nodulation in cultures containing either one or two plants. There was always a predominant number of paired plants, but in spite of this disparity in replication a definite trend was observable in the results.

Of 33 such experiments all but 6 showed a larger number of nodules in the single plant cultures, and of those that did not the number of replicates of the single plant cultures were so small as to make comparison doubtful. These results are summarized in Fig. 1, which shows the distribution of the experiments in terms of percentage reduction in nodulation on paired plant cultures compared with single plant cultures. The experiments inoculated with effective or ineffective strains of bacteria are shown separately on the histogram and it is evident that bacterial strain type has no influence on the distribution.

It might be anticipated that the size attained by individual plants would depend upon the presence of a companion plant, and in all experiments in which dry weights were taken this result was obtained, as shown in Table I. With effective inoculation, plants grown singly were generally more than half as large again as those grown in pairs, independently of the number of nodules formed. With an ineffective strain, on the other hand, reduction in

dry weight was proportionately much less marked although nodulation was considerably depressed. Further data on interaction between ineffectively responding plants will be given in later experiments, but the above data already indicate that reduction in nodule number cannot be fully explained in terms of differences in plant size.

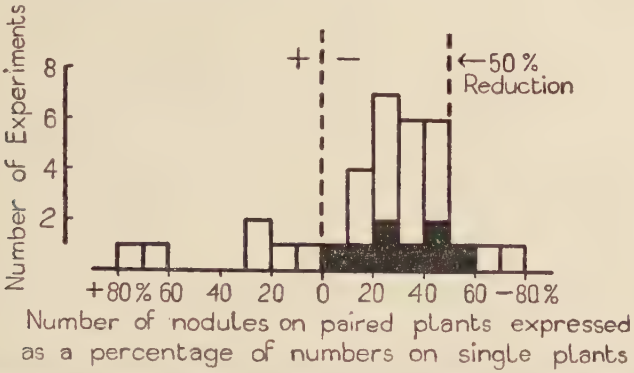

FIG. 1. Reduction in nodule number on plants grown in pairs compared with plants grown singly. Data from 33 experiments comprising 489 single plant cultures and 1,842 cultures of two plants each. □ Inoculated with effective strain; ■ inoculated with ineffective strain.

TABLE I

Percentage Reduction in Number of Nodules and Dry Weight of Clover Plants grown in Pairs as compared with Single Plant Cultures

Strain of Bacteria.	% reduction in nodule number.	% Reduction in dry weight.
Effective	0·6	26·3
,,	17·1	37·1
,,	23·2	32·4
,,	45·4	26·1
,,	72·7	42·4
Ineffective	10·6	19·2
,,	44·3	7·8

CONFIRMATORY EXPERIMENTS ON PLANTING RATE AND VOLUME OF MEDIUM

The first of these experiments was designed to confirm and extend these observations under more standardized conditions, in particular with equal replication and with a controlled volume of medium (10 ml.); each tube was inoculated with an effective strain of bacteria. Observations were made of the time at which nodules first appeared on the roots and nodule counts were made at 6 weeks without removing the plants from the tubes. At harvest (11 weeks) the plants were removed from the tubes and nodules counted and measured; results are shown in Table II.

Nodule formation began at the same time on all sets of plants. Fig. 2 shows the accumulated totals of plants with nodules for each treatment at

progressive stages after the beginning of the experiment; no appreciable differences are shown.

Table II shows that the results of the earlier experiments are confirmed, the number of nodules developed per plant showing a reduction in the presence of another plant. This reduction is already appreciable within 3 weeks of the formation of the first nodules and at 11 weeks the numbers of nodules

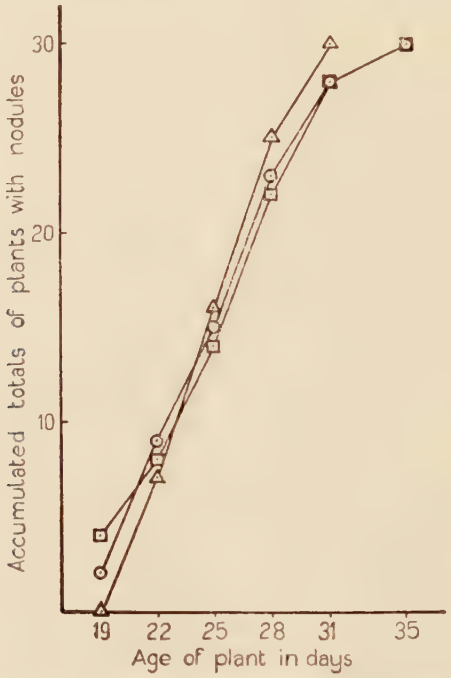

FIG. 2. Progress of nodulation on plants grown singly (⊡), in pairs (⊙), and in triplets (△).

developed per plant were found to be reduced in exact proportion to the number of plants present, so that the number on plants grown in triplets was not significantly different from two-thirds the number on plants grown in pairs, or one-third of the number on a single plant. The data for nodule size is less consistent, a maximum mean nodule length occurring in the treatment giving the fewest nodules and a minimum in the treatment corresponding to an intermediate number of nodules. The relation between nodule size and planting rate is further examined in a later experiment.

The simple inverse relation demonstrated between the number of nodules formed per individual plant and the density of planting in a culture may reflect, as suggested above, the accumulation of a nodule inhibitory substance in the medium. Were this so a reduction in the volume of the medium might be expected to lead to a reduction in nodule number. The influence of the volume of the medium was therefore examined in a factorial experiment combining in all possible ways three rates of planting (1, 2, or 3 plants per

TABLE II

	One plant per tube.	Two plants per tube.	Three plants per tube.
Mean number of days from sowing to the appearance of the first nodule	26·5±0·26	26·4±0·25	26·2±0·18
Mean nodule number per plant at 6 weeks	11·5±1·3	7·9±0·7†	7·0±1·4†
Observed mean number of nodules at 11 weeks . .	29·1±3·1	19·2±5·7	9·6±1·1
Calculated numbers . .		14·5	9·7
Mean nodule length at 11 weeks	9·8±0·15	9·1±0·18	11·3±0·29

† The number of nodules on each plant could not be separately counted at this stage without removing the plants from the tubes, so that the means and S.E. are computed from the tube means and tube variances.

tube), with three volumes of medium (4, 8, 12 ml. per tube). The number of tubes allotted to each treatment was arranged in such a way that the number of seedlings in each treatment was always the same. The watering of each culture was carried out under sterile conditions with half-strength culture solution, sufficient liquid being added at each watering to restore the weight of each tube to its original value. The total amounts so administered varied from 2·9 ml. per tube for the 1-plant/4-ml. treatment to 4·3 ml. for the 3-plant/12-ml. treatment. The time at which nodules first appeared was noted for each plant, the colour and vigour of growth was recorded at 5 weeks, and at harvest (16 weeks) all nodules were counted and measured; results are set out in Table III.

TABLE III

The Influence of Planting Rate and Volume of the Medium on Nodule Formation in Clover

Volume of medium.	1 plant per tube.	2 plants per tube.	3 plants per tube.	Marginal means.
A. Mean number of days from sowing to the development of the first nodule				
4 ml. . .	33·9	36·8	39·1	36·6±1·01
8 ml. . .	35·3	35·5	40·3	37·1±1·01
12 ml. . .	32·0	27·6	30·3	30·2±1·26
Array means .	33·8±1·18	34·0±1·24	36·6±1·01	
B. Mean number of nodules per plant (O, observed; E, expected)				
4 ml. (*O*) .	20·4	9·3	5·3	11·7±1·73
(*E*) .	16·4	8·2	5·5	
8 ml. (*O*) .	34·9	16·1	12·1	21·0±1·73
(*E*) .	32·7	16·4	10·9	
12 ml. (*O*) .	31·6	23·5	14·1	22·8±1·86
(*E*) .	49·1	24·5	16·4	
Array means (*O*)	26·0±1·75	15·4±1·83	10·6±1·72	

Difference between expected and observed values in treatment containing 1 plant in 12 ml. = 16·7±4·55 ($P < 0·01$), remainder N.S.

TABLE III (*cont.*)

Volume of medium.	1 plant per tube.	2 plants per tube.	3 plants per tube.	Marginal means.
C. Nodule length per treatment (mm.)				
4 ml. . .	1·19	1·35	1·33	1·25±0·022
8 ml. . .	0·94	1·00	0·99	0·96±0·016
12 ml. . .	0·90	0·95	1·01	0·94±0·017
Array means .	0·99±0·014	1·06±0·020	1·06±0·023	
D. Colour of youngest open trifoliate leaf matched against Ridgway colour standard mean 'Degrees of blackness' at 5 weeks				
4 ml. . .	0·33	1·67	1·33	1·11
8 ml. . .	−1·83	−1·50	0·50	−0·94
12 ml. . .	−3·36	−1·50	−1·33	−2·12
Array means .	−1·60	−0·63	0·16	
At 16 weeks				
4 ml. . .	1·58	1·42	0·93	1·31
8 ml. . .	1·58	2·00	1·42	1·67
12 ml. . .	0·09	1·88	2·09	1·32
Array means .	1·11	1·60	1·47	

In this experiment the onset of nodulation was little affected by plant density, the earliest formation of nodules tending to occur on single plant cultures. The largest volume of medium, however, promoted significantly earlier initiation of nodules than did the other two volumes used. The earlier formation of nodules in the larger volume of medium was evident at an early stage as shown in Fig. 3.

The data for nodule number shows two trends; as in previous experiments nodule formation is reduced by the presence of a companion plant, and it is also less in the smaller volumes of media. These trends are simply related to each other, a single plant in 4-ml. medium forming about the same number of nodules as one of a pair in 8-ml. medium or one of three in 12-ml. medium. The number of nodules per plant per ml. tends, therefore, to a constant value, and on this basis the expected numbers of nodules (E) have been calculated for each treatment and may be compared with numbers of nodules actually observed (O). Only the treatment containing a single plant in the largest volume of medium shows a significant deviation from the expected value. Here the observed number is much less than expectation, suggesting that there is a maximal number of nodules possible for plants of this age and that this number has been reached. This conclusion is supported by the fact that the nodule numbers on single plants in 12 ml. and 8 ml. are not significantly different, whereas both greatly exceed the number formed by 1 plant in 4 ml.

The previous experiment indicated that the fewer nodules formed on plants sown as triplets were of the largest average size. This result is confirmed although the differences are small. The influence of volume is here more

marked than planting rate, an increase in volume leading to a decrease in nodule size. The results of both these experiments illustrate the well-known inverse relationship concerning nodule size and number (see Fred, Baldwin, and McCoy, 1932).

In the absence of combined nitrogen in the medium, the seedlings pass through a phase of nitrogen deficiency until the first-formed nodules begin

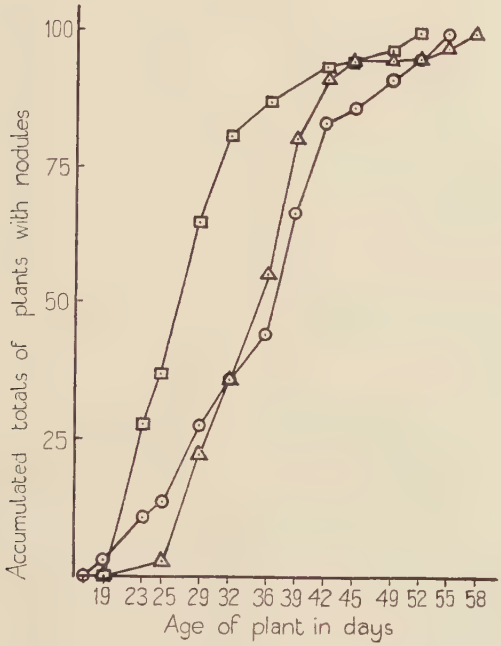

FIG. 3. Progress of nodulation on plants grown in 4 ml. (△), 8 ml. (⊙), and 12 ml. (⊡) of medium. (As percentage of maximum.)

to function. Then the colour of the leaves change from yellow-green to dark green, the petioles losing anthocyanin pigment. The stage at which nitrogen first becomes available was followed by observing the changes in leaf colour by matching the colour of the youngest open trifoliate leaf against Ridgway's colour standards. No consistent trends were noted in the average 'spectral colours', but very marked differences were noted in the 'degrees of blackness' observed at 5 weeks, as shown in Table III. An increase in planting rate or a decrease in volume both led to a pronounced increase in intensity of colour; i.e. to a proportionate decrease in the amount of 'white pigment' in the leaf. The data in the table are stated in conventional manner, assigning positive unit values to each of the shades 'i', 'k', and 'm' and negative unit values to the scale of tints 'b', 'd', and 'f'; Ridgway's manual (Ridgway, 1912) should be consulted for details.

The deeper green colour of plants in the smaller volumes of media and at

the higher rates of planting may be due to differences in the rates of fixation or utilization of nitrogen fixed in the young nodules. It is not due to earlier formation of nodules as the data in Table III and Fig. 3 show, but may reflect differences in nodule growth-rate or activity. Measurements of nodule length lend support to this suggestion; where nodules continue to be produced in large numbers the development of the individual nodule may be slower, and this may prolong the initial period of nitrogen starvation. At 16 weeks all differences in colour of plants had vanished.

This experiment establishes the mode of interaction between plants but cannot discriminate whether the effect is due to competition or inhibition. The direct study of competition within a bacteriologically controlled vessel of standard size is difficult to undertake, the more so as the growth of the nodulated plant is itself determined largely by the activity of nodules in fixing atmospheric nitrogen. Accordingly in the following experiment the volume and composition of the culture medium has not been experimentally varied, but the competition between plants has been considerably altered by the use of contrasting plant types and bacterial strains. By this means it was hoped to distinguish between competitive and inhibitory effects. The following different kinds of plants were used in these studies:

1. Normal effectively responding clover plants.
2. Clover inoculated with ineffective strains of bacteria.
3. Selected lines of clover which respond ineffectively with a normally effective strain of bacteria.
4. Abundantly and sparsely nodulating lines of plant.
5. Plants which start to form nodules early or late in development.
6. Selected lines of clover which are completely resistant to infection by clover nodule bacteria.
7. Plants belonging to different cross-inoculation groups.
8. Non-leguminous plants.

Interaction between Effectively and Ineffectively Responding Plants

It has already been shown in the miscellaneous experiments reported above that clover plants inoculated with ineffective strains of bacteria mutually suppress each other's nodulation when grown together. Such plants make little growth and probably do not appreciably shade each other nor affect the quantity or balance of the salts in the medium. An experiment designed specifically to test strain differences in interaction has confirmed this result. The mean values for nodule number and root number shown in Table IV refer to the logarithms of the original counts (see previous paper for relation between variance and mean in original and transformed data; Nutman, 1952).

With either effective or ineffective inoculation, nodule number and root number are significantly depressed among paired plants compared with plants grown singly. The statistical interaction between association (i.e. one versus two plants) $\times$ bacterial strain for nodule number is not significant, showing

that the degree of inhibition is proportionally the same with effective and ineffective inoculation. For root number, however, the association × bacterial interaction is significant, a more marked reduction of root formation occurring in the paired cultures inoculated with effective strains of bacteria. This result is anticipated since the ineffective plants produce very few roots whereas root formation on effective plants increases as soon as the nitrogen fixed in the early nodules becomes available.

TABLE IV

The Interaction of Plants inoculated with Effective and Ineffective Strains of Bacteria

Companion plant.	Effective inoculation		Ineffective inoculation	
	Mean log. nodule No.	Mean log. root No.	Mean log. nodule No.	Mean log. root No.
None . .	1·59	1·72	1·91	1·40
One . .	1·39	1·38	1·79	1·23

Interaction: Association (plant number per culture) × Bacteria
(i) for nodule number $0·08 \pm 0·062$ N.S.
(ii) for root number $0·17 \pm 0·057$ ($P < 0·01$).

Interaction among ineffectively responding plants and between plants of contrasted response has also been examined using bred lines of clover which form a wholly ineffective symbiosis with a normally effective strain of bacteria (Nutman, 1949*a*). Two small experiments were carried out with this material; Table V shows mean nodule number and dry weight at harvest in each experiment. The mean log. values of nodule number and dry weight are shown

TABLE V

Interaction between Effectively and Ineffectively Responding Plants

Companion plant.	Mean nodule number ($\log n+1$) on:				Mean dry weight (log.) of:			
	Effective plant		Ineffective plant		Effective plant		Ineffective plant	
	Expt. 1.	Expt. 2.	Expt. 1.	Expt. 2.	Expt. 1.	Expt. 2.	Expt. 1.	Expt. 2.
None (*A*)	1·511	1·445	1·850	1·710	1·650	1·757	1·105	1·322
Effective plant (*B*)	1·361	1·314	1·565	1·768	1·522	1·467	1·115	1·894
Ineffective plant (*C*)	1·040	1·310	1·710	1·666	1·450	1·562	1·045	1·095
Tests of significance of mean differences on combined data:								
A—*B* . .	$0·143 \pm 0·062$*		$0·091 \pm 0·148$		$0·182 \pm 0·060$**		$0·326 \pm 0·095$	
A—*C* . .	$0·330 \pm 0·127$**		$0·073 \pm 0·123$		$0·197 \pm 0·091$		$0·210 \pm 0·078$*	
B—*C* . .	$0·159 \pm 0·115$		$0·028 \pm 0·123$		$-0·054 \pm 0·075$		$0·165 \pm 0·072$*	

* $P = 0·05$. ** $P < 0·01$

for effective and ineffective plants when growing alone (*A*), i.e. with no companion plant, or in the presence of an effectively responding plant (*B*) or an ineffectively responding plant (*C*).

Owing to limited replication (in some cases only two) some of the differences already demonstrated do not reach a high level of significance, e.g. depression in nodule formation following pairing of effectively responding plants. Effectively and ineffectively responding companion plants are, however, equally inhibitory to nodule formation on effectively responding plants. The differences which appear in the numbers of nodules formed on ineffective plants when growing alone or in the presence of effective or ineffective companion plants are in the same sense but do not, however, reach a level of significance; a more adequately replicated experiment would be required to establish these effects beyond doubt.

Plant size in the various treatments in general follow nodule number. In particular it should be noted that an ineffectively responding plant is as influential in depressing growth of an effectively responding as a second effectively responding plant.

Interaction between Abundantly and Sparsely Nodulating Plants

It was shown in the earlier papers of this series that clover plants differ among themselves in relative susceptibility to infection. These differences are determined by hereditary factors in the plant which also effect in the same way the number of lateral roots formed. Abundantly and sparsely nodulating and rooting plants may differ in the absolute numbers of lateral meristematic foci each is able to form or in the inhibiting propensities of the root and nodule meristems. In the latter event sparsely nodulating plants may be expected to have a stronger inhibiting action on the nodulation of a companion plant.

Two experiments were set up to investigate the interaction of sparsely and abundantly nodulating plants using three families in the first experiment and five other families in the second. The plants of each family were grown singly and in pairs in all combinations. Results are given in Tables VI and VII, in which the families of test plants are arranged from left to right in order of increasing susceptibility, the families of companion plants being arranged in the same vertical order. The entries in any one column always refer to the behaviour of the plants belonging to the family specified at the head of the column, either when growing alone or in the presence of a member of its own or any other family; whereas in the rows the entries show the behaviour of the plants belonging to each family in turn, either alone or in the presence of a companion plant drawn from the family shown in the first column under the heading 'companion plant'.

It is clear from these results that sparsely and abundantly nodulating plants do not significantly differ although a trend is evident. In both experiments the least abundantly nodulated companion plant has had least effect on nodulation, but in neither case does this difference reach a level of significance.

(Linear regression coefficients of log. nodule number on test plant against log. nodule number on companion plants are $0{\cdot}200 \pm 0{\cdot}673$ and $0{\cdot}077 \pm 0{\cdot}302$.) The average reduction in nodulation due to pairing is also shown in Tables VI and VII; these show no regressions with family means, indicating that on the untransformed data the reduction on pairing is proportional to the number of nodules formed. It may be concluded from these results that neither the inhibitory activity of a plant nor its sensitivity to inhibition are related to its inherent susceptibility to infection.

Table VI

Interaction between Abundantly and Sparsely Nodulating Plants: Nodules per Plant. Mean (log $n+1$)

Companion plant.	Family No. 1.	Family No. 2.	Family No. 3.	Marginal means.
None . .	1·48	1·54	1·77	1·595±0·0218
Family No. 1 .	1·30	1·34	1·57	1·404±0·0305
Family No. 2 .	1·21	1·29	1·48	1·327±0·0288
Family No. 3 .	1·24	1·28	1·44	1·319±0·0339
Array means .	1·308 ±0·0305	1·361 ±0·0288	1·580 ±0·0339	(Number of replicated plants = 122)
Mean reduction due to pairing	0·23	0·24	0·20	

Difference between Family No. 1 and Family No. 3 as companion plants:
$0{\cdot}085 \pm 0{\cdot}046$
$t = 1{\cdot}85$ N.S.

Table VII

Interaction between Sparsely and Abundantly Nodulating Plants: Nodules per Plant. Mean (log $n+1$)

Companion plant family.	Family No. 1.	Family No. 2.	Family No. 3.	Family No. 4.	Family No. 5.	Marginal means.
None . .	1·43	1·58	1·43	2·00	2·08	1·704±0·0339
No. 1 . .	1·16	1·27	1·44	1·80	1·84	1·502±0·0338
No. 2 . .	1·05	1·32	1·42	1·67	1·97	1·486±0·0381
No. 3 . .	1·07	1·01	1·41	1·65	1·84	1·487±0·0392
No. 4 . .	1·20	1·18	1·21	1·61	1·81	1·402±0·0409
No. 5 . .	1·18	1·18	1·36	1·83	1·83	1·474±0·0371
Array means	1·179 ±0·0338	1·257 ±0·0381	1·378 ±0·0392	1·762 ±0·0409	1·895 ±0·0371	(Number of replicated plants = 218)
Mean reduction due to pairing	0·30	0·39	0·06	0·29	0·22	

Maximum difference in inhibition by companion plants (families 1 and 4)
$= 0{\cdot}100 \pm 0{\cdot}0531$, $t = 1{\cdot}88$ N.S.

Interaction between Early and Late Nodulating Plants

The clover seedling is not normally susceptible to infection until about the third week of growth. Considerable individual variation occurs in this respect, some plants forming nodules as early as 10 days from sowing and others as late as 5 or 6 weeks. These differences in nodulation time are hereditarily determined and by simple selection early and late nodulating lines may be raised.

The experiments of Thornton (1929) and Ludwig and Allison (1935) have shown that the infection of the lucerne seedling is stimulated by the presence of older lucerne plants. It was suggested that a nodule initiating substance is normally secreted by the root. In view of this finding it is of special interest to determine the mutual influence of early and late nodulating lines which presumably differ in their response to, or production of, the nodule initiating substance postulated by Thornton.

Eight families of clover were selected for experimentation, two families nodulating early, two nodulating late, and four nodulating at intermediate times. As in previous experiments the plants of these families were grown singly and in pairs in all combinations. The time at which nodules first appeared was noted for each plant and after a period of about 100 days' growth the number of nodules, number of roots, and the dry weight of each plant was determined. The mean time (days from sowing) at which nodules appeared on each family of plants are shown in Table VIII as well as the mean nodulation times of all families in the presence of a member of each family in turn.

Table VIII

Average Times of Primary Infection in Selected Early and Late Nodulating Lines of Clover

Family:	Nodulation times for each family.		Family:	Nodulation times for all families in presence of each family in turn as companion plant.	
No. 1	14·52±0·88	15·40±0·59	No. 1	19·95±1·05	18·93±0·71
2	16·12±0·95		2	18·91±0·95	
3	18·65±0·75	19·10±0·43	3	18·96±0·92	19·23±0·52
4	18·91±0·98		4	19·76±1·11	
5	19·43±0·71		5	19·70±0·88	
6	19·57±1·22		6	17·56±1·53	
7	22·93±0·88	23·35±0·62	7	19·25±1·14	19·81±0·74
8	23·78±0·88		8	20·23±0·98	

Influence of companion plant on infection:

With no companion plant . .	18·77 days
With companion plant . .	19·29 days

Difference 0·52±0·65 N.S.

The arrangement of this experiment was essentially the same as that shown in Table VII, now using selected early and late nodulating lines in place of sparse and abundant lines, but in Table VIII only the marginal means for rows and columns are shown. Thus entries in the first column show the mean nodulation times *of plants all belonging to any one family* either growing alone or in the presence of a member of its own family or a member drawn from each of the other families in turn. Each entry in the second column shows the mean nodulation time for *members of various families* in the presence of a companion plant of the family indicated.

The families, as already stated, were selected for different times of nodulation, and as can be seen from the entries in column 1 this characteristic of these families appears just as markedly in the presence of a companion plant belonging to each of the other families in turn. In column 2, on the other hand, in which the mean nodulation times for members of all families in the presence of a companion plant drawn from one of the families as indicated, shows a completely different result. For here, as would be expected, if there is no interaction, the mean time to nodulate approaches a constant value irrespective of the characteristic of the family from which the companion plant is drawn. The overall effect of the presence or absence of a companion plant on time to nodulation is also entered in Table VIII and shows no effect, the difference being entirely insignificant.

This latter result confirms previous observations that nodulation is not advanced in paired clover cultures, and thus differs from the result obtained by Thornton with lucerne, although it should be noted that the stimulation of nodulation on lucerne was obtained with older plants growing among young seedlings and not as here with plants of the same age. It will be shown later that lucerne does, in fact, differ from clover in this respect.

The absence of any interaction between plants with respect to nodulation time enables a direct examination to be made of the relation between nodulation time and the number of nodules produced and other characteristics. This may be examined as before on the basis of means for families, or in terms of the actual times of primary infection noted on individual plants; the latter procedure enables this relation to be investigated over a wider range of nodulation time, viz. 10–30 days in place of 14–24 days.

The mean log. number of nodules, lateral roots, and dry weight are shown in Table IX, using both these terms of reference. In the upper half of the table mean values are given for the data arranged either as families or classes of plants differing in times of nodulation. In the lower part of the table the influence of these different kinds of plant is recorded in reducing nodule formation, &c., in paired cultures.

The nodule numbers produced by the families nodulating at progressively later times (*A*) shows little trend for one plant per tube, but for two plants per tube there is some indication of an increase in nodule number in the earlier families. A similar trend is seen more markedly in the number of lateral roots produced, while the dry weight shows little consistency. When

Table IX

Nodule Formation, Lateral Root Formation, and Dry Weights

A. Families forming nodules at progressively later times in seedling development

Family No.	Log. nod. No. +1		Log. lat. rt. No. +1		Log. dry wt.		Days to nodulation.
	1 ppt.†	2 ppt.	1 ppt.	2 ppt.	1 ppt.	2 ppt.	
1	1·665	1·496	1·938	1·642	1·862	1·671	10
2	1·638	1·360	1·758	1·471	1·914	1·642	12
3	1·805	1·270	1·695	1·461	1·837	1·567	15
4	1·545	1·483	1·535	1·475	1·880	1·620	18
5	1·460	1·070	1·513	1·180	1·880	1·539	20
6	1·586	1·310	1·572	1·480	1·823	1·573	22
7	1·560	1·295	1·617	1·311	1·853	1·474	24
8	1·670	1·300	1·630	1·370	1·895	1·524	26
							30

B. Individual plants (means) forming nodules at progressively later stages in development

Log. nod. No. +1		Log. lat. rt. No. +1		Log. dry wt.	
1 ppt.	2 ppt.	1 ppt.	2 ppt.	1 ppt.	2 ppt.
1·626	1·296	1·790	1·553	1·780	1·624
1·527	1·442	1·645	1·558	1·852	1·732
1·512	1·396	1·712	1·453	1·923	1·610
1·578	1·422	1·603	1·516	1·856	1·598
1·574	1·342	1·774	1·449	1·860	1·581
1·561	1·241	1·707	1·475	1·827	1·590
1·397	1·266	—	1·407	—	1·589
1·498	1·051	1·510	1·242	1·840	1·457
1·390	1·078	1·490	1·079	1·835	1·304

† ppt = plant per tube.

Interaction between Early and Late Nodulating Plants, with respect to Nodule Formation (N) and Lateral Root (R) and Dry Weight (Dwt.), determined A, for Families, and B for Individual Plants

A. Family of companion plant.	log $N+1$.	log $R+1$.	log dwt.
1	1·346	1·431	1·548
2	1·285	1·442	1·548
3	1·324	1·470	1·585
4	1·277	1·359	1·539
5	1·302	1·375	1·622
6	1·366	1·392	1·567
7	1·344	1·610	1·656
8	1·332	1·448	1·594

B. Days to nodulation on companion plant.	log $N+1$.	log $R+1$.	log dwt.
10	1·090	1·256	1·469
12	1·290	1·447	1·526
15	1·224	1·506	1·591
18	1·412	1·535	1·640
20	1·308	1·401	1·566
22	1·308	1·403	1·528
24	1·366	1·477	1·581
26	1·329	1·367	1·602
30	1·400	1·498	1·710

the data, however, are arranged into classes of individuals with progressively later nodulation (*B*) the trends in nodule number, lateral root production, and dry weight are much clearer and indicate in every case an inverse relation with time to nodulation.

On the other hand, as shown in the lower part of the table, members of early nodulating companion families of plants show some sign of causing

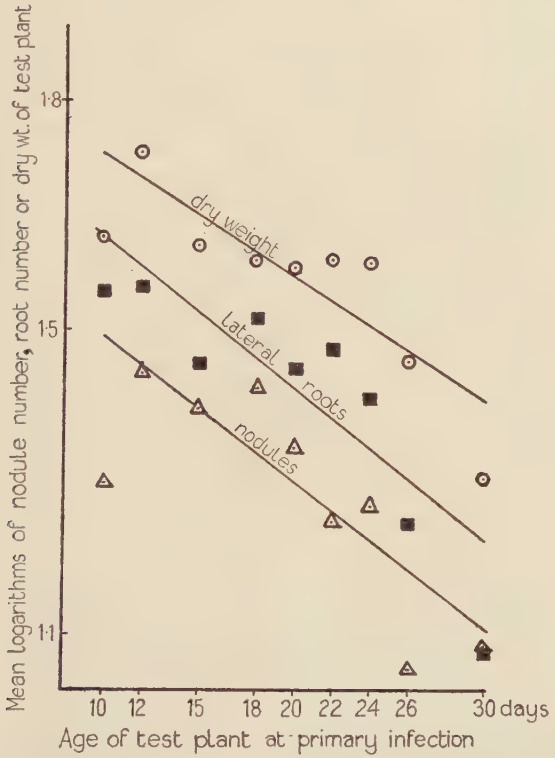

FIG. 4. Linear regressions of logarithms of nodule number (△), logarithm of lateral root number (■), and logarithm of dry weight (⊙) on time of primary infection on test plant.

a larger reduction in nodule number, root number, and the dry weight of plants with which they are grown than do late nodulating companion plants —a relation which becomes quite evident when the data are arranged in terms of classes of progressively later nodulating companion plants. These relationships have been analysed for the data based on individual nodulation times and are figured in Figs. 4 and 5 and in Table X. It should be noted that the mean values corresponding to the earliest and latest times of infection were computed from few observations, whereas the intermediate ones are based upon a large number of counts. In calculating the linear regressions drawn through the observed means in Figs. 4 and 5 and tabulated in Table X, due allowance has been made for inequalities in replication by appropriate weighting.

The regression lines for nodule number, root number, and dry weight incline downwards when plotted against the time to nodulation of the test plant (Fig. 4) but show an ascending slope with increasing nodulation times of the companion plant (Fig. 5). The regression coefficients with respect to nodulation time of the test plant are therefore negative in value for nodules, roots, and dry weight and are statistically highly significant. The stronger inhibitory

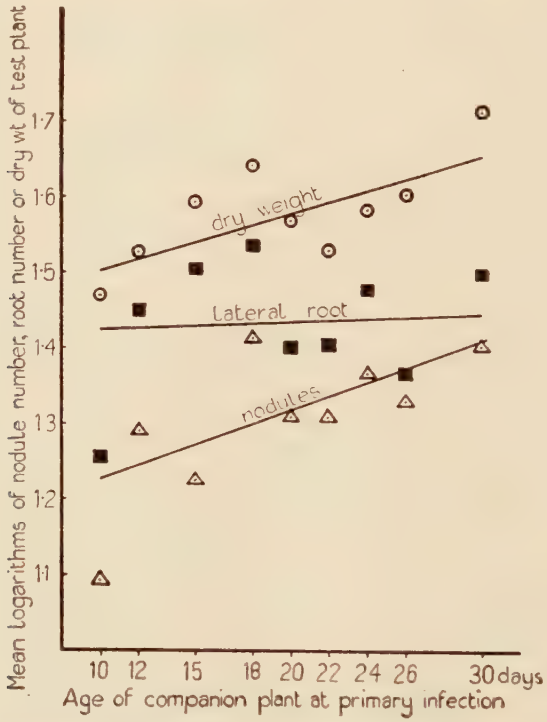

FIG. 5. Linear regressions of logarithm of nodule number (△), logarithm of lateral root number (■), and logarithm of dry weight (⊙) on time of primary infection on companion plant.

TABLE X

Relation between the Number of Nodules, Lateral Roots, and Dry Weight and the Time at which Nodules first form on (A) Test Plant and (B) on the Companion Plant

	Linear Regression Coefficients		
	Nodules.	Lateral roots.	Dry weight.
(A)	*On nodulation time of test plant*		
	$-0{\cdot}0190 \pm 0{\cdot}0040^{**}$	$-0{\cdot}0205 \pm 0{\cdot}0040^{**}$	$-0{\cdot}0164 \pm 0{\cdot}0018^{**}$
(B)	*On nodulation time of companion plant*		
	$+0{\cdot}0086 \pm 0{\cdot}0040^{*}$	$+0{\cdot}0010 \pm 0{\cdot}0040$	$+0{\cdot}0073 \pm 0{\cdot}0018^{**}$

* $P < 0{\cdot}05$. **$P < 0{\cdot}01$.

activity of early nodulating plants is indicated, on the other hand, by positive regression coefficients which are significant for nodules and dry weight but do not reach a level of significance for roots.

That early nodulating plants themselves produce more nodules and roots and grow to a greater size may be due to the longer time available for nitrogen fixation, &c., before the end of the experiment compared with plants whose

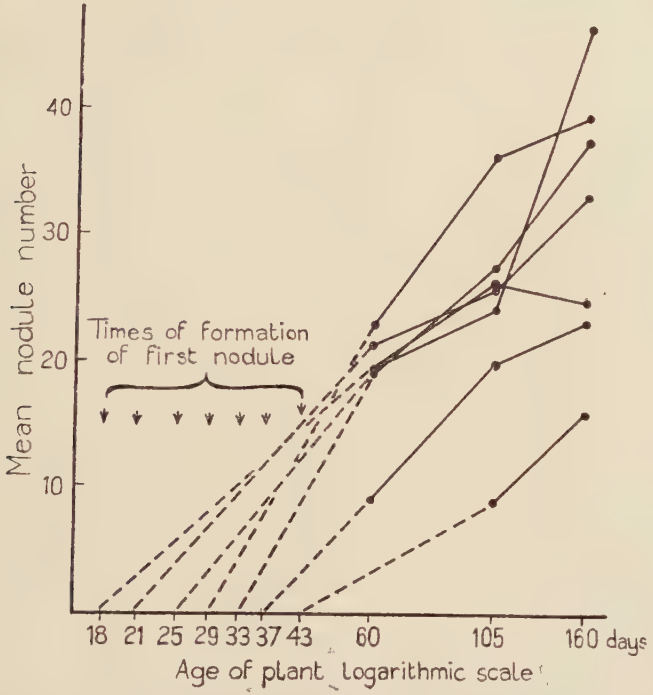

FIG. 6. Rates of nodule formation on plants which begin to form nodules at early or at later stages of development.

development is retarded by a delayed nodule development. Corroboration for this suggestion is provided by the results of a previously conducted experiment in which plants of these families were set up in single cultures and harvested at intervals over a period of 160 days. The results in Fig. 6 show that the rates of infection on a log. time basis are approximately the same for early and late nodulating plants.

It would appear from the results shown in Table X that the early nodulating plants are more active in the production of nodule and root inhibitors. With abnormally early or late material inhibition may account for as much as a 50 per cent. difference in the actual number of nodules formed. In this experiment, therefore, there is a clear correlation between the numbers of nodules produced on the companion plant and the amount of inhibition obtained. This result may be compared with the experiment using families

selected for abundant or sparse nodulation independently of nodulation time (see Tables VI and VII). There, also, the plants selected for abundance of nodules caused maximum inhibition, although not to a significant degree. This effect may be due also to differences in nodulation time. It is possible, however, to select and breed for each character separately, indicating loose genetic linkage, though the slight degree of correspondence between these two experiments conducted with material selected on one hand for nodulation time and on the other for nodule number may be attributed to genetic linkage. It would be of interest in this connexion to determine the inhibitory activity of lines of plants selected either for early and abundant nodulation or for late and sparse nodulation.

Interaction Between Resistant and Susceptible Plants

Clover plants exhibit great variability in relative susceptibility both with regard to nodulation time and actual number of nodules formed. In addition, clover may be completely resistant to infection by its own strain of bacteria. Resistance of this kind is also hereditarily determined and in the families to be used in these experiments is due to a simple Mendelian factor associated with a cytoplasmically transmitted component (Nutman, 1949) and is thus genetically distinct from the relative resistance of sparsely or late nodulating lines.

Experiments on the interaction of resistant and susceptible plants were carried out with families which segregated resistant and susceptible plants in the proportion 1:3. In this way susceptible and resistant plants differed in a single gene only. In the first experiment eight families segregating resistant plants were sown singly, in pairs, and in threes, the numbers of tubes planted in each set being adjusted to reduce as far as possible the unavoidable disparity in numbers of replicates per treatment. Inoculation of the experiment was made from an effective subculture of bacteria and after 98 days' growth the nodules were counted.

The results are summarized in Table XI, expt. 1, and show that both resistant and susceptible plants depress nodule formation on a companion plant. On the assumption that resistant and susceptible plants are equally inhibitory the differences between observed and expected values are also shown. These show that the slightly stronger inhibition obtained in the presence of a resistant compared with a susceptible plant is not significant. However, in view of the importance which might attach to any differences between these plants in inhibition, a second experiment was set up in which six families segregating resistant plants (1:3) were used, the plants being sown singly or in pairs only but with increased replication. The results are shown in Table XI, expt. 2, and confirm the result of the previous experiment; resistant and susceptible plants equally inhibiting nodulation on an associated plant. The number of roots on the susceptible plant is also affected by the presence of a resistant plant, but to a significantly smaller extent than with another susceptible plant. On the resistant plants themselves the number of

roots was markedly and equally depressed by the presence of either a resistant or susceptible companion plant. These experiments show that resistant and susceptible plants are similar in their interactions, although the growth of the resistant plant is strictly limited by the reserves in the seed.

TABLE XI

Interaction between Susceptible and Resistant Plants

Companion plant.	Experiment 1 Mean log. No. nodules (+1) on susceptible plants.	Difference between observed and expected log. nodule Nos.
None	1·520	—
One susceptible (sus.)	1·368	—
Two sus.	1·235	—
One resistant (res.)	1·326	0·042±0·074 N.S.
Two res.	1·204	0·035±0·048 N.S.
One res.+one sus.	1·174	0·061±0·117 N.S.
	Experiment 2	
	Mean log. nodule No.	Mean log. root No.
None	1·75	1·56
One sus.	1·59	1·31
One res.	1·56	1·47
	Mean Differences	
1 sus.–2 sus.	0·16±0·031**	0·25±0·028**
1 sus.–(1 sus. with 1 res.)	0·19±0·050**	0·09±0·045*
2 sus.–(1 sus. with 1 res.)	0·03±0·049	−0·16±0·044

* $P < 0·05$. ** $P < 0·01$.

INTERACTION BETWEEN PLANTS OF THE CLOVER, PEA, AND MEDICAGO CROSS-INOCULATION GROUPS

Three separate experiments were carried out on the interaction between members of different cross-inoculation groups. The first between clover and lucerne in which both host plants were sown singly and in pairs, the cultures sown with both clover and lucerne receiving an inoculation of either an effective strain of clover nodule bacteria, or an effective lucerne strain, or both strains. The second and third experiments were set up with three host plants: clover, lucerne, and a small seeded vetch (*Vicia hirsuta*). All plants were sown in pairs in all possible combinations and were inoculated as before with single or with appropriate pairs of bacterial strains.

The shoot of the vetch seedling tended to elongate rapidly and grow into the cotton-wool plug and to become entangled. Accordingly at intervals each shoot apex was carefully bent over and pushed to the bottom of the tube so that at the end of an experiment the top of each vetch plant consisted of three or four loops of stem bearing leaves all along its length.

In each experiment observations were made at intervals of 2 or 3 days to determine the time at which primary infection took place. Variation about

the mean nodulation time was least on clover and greatest on lucerne, some of the individual plants of the latter remaining un-nodulated for as long as 8 weeks. Nodulation time on each host was distributed normally, nodules forming earlier on clover than on lucerne or vetch.

In Table XII the average nodulation time for each treatment of each experiment is shown as well as the advance (+) or delay (−) in nodulation time due to the association of plants in pairs. The latter have been calculated from the combined results of the three experiments with appropriate weighting. The data show that in most cases nodules form a little earlier in plants in pairs, twelve of the fifteen comparisons giving positive values for the difference between mean nodulating time of single or paired plants. None of these individual differences, however, reaches a level of significance, and only in lucerne as the test plant combining all the data does the advance in average nodulation time become appreciable ($P = 0{\cdot}05$). This result confirms the suggestion made by Thornton that the lucerne plant is sensitive to a nodule-stimulating root secretion, and it would appear that a companion clover or vetch plant acts in the same way. So far as clover is concerned a companion

Table XII

Average Number of Days to Primary Infection in Clover, Lucerne, and Vetch, grown singly and in association

Symbols: Plants—C, clover; L, lucerne; V, vetch.
Bacteria—c, clover strain; l, lucerne strain; v, vetch strain.

Plant association.	Bacterial strain.	Expt. 1.	Expt. 2.	Expt. 3.	Mean difference (singles–pairs).	Average mean differences (weighted).
C alone	c	18·35	26·11	19·77	—	
C in pairs	c	19·16	26·71	17·85	−0·466±0·534	
C with L	c	18·25	19·25	17·68	+0·648±0·630	
C „ L	c+l	18·00	22·17	19·77	+0·406±0·648	+0·2036±0·3305
C „ V	c	—	25·08	17·20	+2·201±1·702	
C „ V	c+v	—	25·20	17·35	+0·346±1·645	
L alone	l	33·78	31·67	31·09	—	
L in pairs	l	30·37	35·63	25·91	+2·727±2·139	
L with C	l	31·83	25·12	35·81	+1·758±2·356	
L „ C	l+c	32·46	34·75	28·08	+1·288±2·815	+2·5953*±1·1947
L „ V	l	—	31·00	23·46	+5·076±3·659	
L „ V	l+v	—	34·50	24·85	+3·602±3·315	
V alone	v	—	46·00	15·10	—	
V in pairs	v	—	38·11	14·02	+1·346±1·478	
V with C	v	—	31·43	23·30	+5·921±3·651	
V „ C	v+c	—	43·60	15·96	−0·728±2·517	+1·1164±0·8935
V „ L	v	—	44·00	13·55	+1·568±1·750	
V „ L	v+l	—	29·93	16·68	−0·252±2·062	

* $P < 0{\cdot}05$.

Difference between stimulating effects of inoculated and uninoculated companion plants 1·579±1·205 N.S.

Host differences in stimulation: C, +1·253±0·945; L, +1·489±0·692; V, +2·006±0·891.

plant shows signs of *delaying* nodulation as in the previously reported experiments (see Tables II, III, and VII), although this effect is nowhere significant, while a companion lucerne or vetch plant leads to earlier nodulation, but again not significantly.

The average degrees of stimulation shown by the three host plants are given at the bottom of the table as well as a comparison of the stimulating effects of inoculated and uninoculated companion plants: none of these differences reaches a level of significance.

Nodule numbers were determined in each experiment, dry weights for two of the experiments, and root numbers for the final experiment. For the last experiment also the pH of the medium was taken at harvest and counts were made by Miss Hilary Purchase of the numbers of bacteria per ml. in the fluid part of the medium for the treatments planted with clover or lucerne or both of these host plants.

Treatment means for nodules, root number, and dry weight are set out in Table XIII and a general asnalyis for all three experiments in Table XIV. In these experiments a large number of comparisons may be made, but those of most interest concern the inhibition produced by different host plants in the presence or absence of their own strains of bacteria. It may be recalled that uninoculated plants make little or no growth and in this respect may be compared with resistant or ineffectively responding clover plants in earlier experiments.

The results in Table XIV show that there are no simple relationships governing the interaction of different species of legume. With few exceptions a smaller number of nodules or roots and a smaller dry weight per plant is found in cultures containing two plants, but only in about half the treatments do these decreases become significant, and it is clear that those that do so are not distributed at random.

Among plants of any one species inhibition is general and effects alike nodules, root formation, and dry-weight increase and is of comparable magnitude. Interactions between host species, however, show no such regularity. Clover, for example, differs from the other hosts in two respects: (1) nodule formation *on* clover is strongly inhibited by all host species whether or not the latter are inoculated (earlier experiments with resistant clover show that this relationship also applies to un-nodulated clover as a companion plant), and (2) inhibitor production *by* clover plants is very much more marked when the clover is itself inoculated, when it effects growth and root development as well as the number of nodules formed on the associated plant. The clover is thus more generally susceptible to inhibition of its own nodules and when inoculated may be presumed to be a potent source of inhibitor. Lucerne, on the other hand, strongly inhibits nodulation on clover and on vetch when it is either inoculated or uninoculated, but it depresses rooting and dry weight to a lesser degree; root formation on clover and vetch even showing a significant increase in the presence of un-nodulated lucerne. In contrast to clover, lucerne is also inhibited to very different degrees by the other host

Table XIII

Cross-inoculation Group Interactions: Table of Means

Symbols: Plants—C, clover; L, lucerne; V, vetch.
Bacteria—c, clover strain; l, lucerne strain; v, vetch strain.

Plant association.	Bacterial strain.	Mean log. nodule No. (+1)					Mean log. root No. (+1)	Mean log. dry wt. mg.	
		Expt. 1, clover family 1.	Expt. 1, clover family 2.	Expt. 1, clover family 3.	Expt. 2.	Expt. 3.	Expt. 3.	Expt. 2.	Expt. 3.
C alone	c	1·482	1·536	1·768	1·426	1·705	1·743	1·477	1·861
CC	c	1·302	1·289	1·443	1·157	1·363	1·500	1·368	1·588
C with L	c	1·268	1·351	1·648	spoilt	1·521	1·929	spoilt	1·835
C ,, L	c+l	1·307	1·338	1·545	spoilt	1·523	1·650	spoilt	1·770
C ,, V	c	—	—	—	0·942	1·060	1·752	1·398	1·844
C ,, V	c+v	—	—	—	1·198	1·630	1·666	1·302	1·951
L alone	l	1·210	1·210	1·210	1·146	1·204	1·765	1·518	1·799
LL	l	0·905	0·905	0·905	0·871	1·041	1·517	1·408	1·502
L with C	l	1·127	1·310	1·151	spoilt	1·309	1·629	spoilt	1·573
L ,, C	l+c	0·828	0·795	1·132	spoilt	0·773	1·207	spoilt	1·280
L ,, V	l	—	—	—	1·195	1·228	1·847	1·632	1·774
L ,, V	l+v	—	—	—	1·077	1·110	1·636	1·437	1·563
V alone	v	—	—	—	0·986	1·646	1·203	1·304	1·836
VV	v	—	—	—	1·147	1·326	1·000	1·254	1·565
V with C	v	—	—	—	0·947	1·564	1·219	1·295	1·667
V ,, C	v+c	—	—	—	0·694	1·092	0·986	1·182	1·410
V ,, L	v	—	—	—	0·702	1·405	1·376	1·762	1·760
V ,, L	v+l	—	—	—	1·213	1·172	1·023	1·363	1·447

Table XIV

Summary of Interactions between Clover, Lucerne, and Vetch

Effect of pairing on nodulation, rooting, and growth. Mean log. differences between paired and single cultures; *positive sign denoting inhibition and negative sign and italic type stimulation. Highly significant differences ($P < 0.01$) in heavy type, less highly significant differences ($P = 0.05$–0.01) in intermediate type.*

Companion plant.	Clover			Lucerne			Vetch		
	Nodules.	Roots.	Dry wt.	Nodules.	Roots.	Dry wt.	Nodules.	Roots.	Dry wt.
Clover: uninoculated	—	—	—	+0·010 ±0·063	+0·136 ±0·145	+0·162 ±0·092	+0·072 ±0·062	*−0·088* *±0·074*	+0·126 ±0·052
inoculated	**+0·268** ±0·029	**+0·243** ±0·055	**+0·253** ±0·030	**+0·325** ±0·064	**+0·558** ±0·160	**+0·239** ±0·076	**+0·500** ±0·062	**+0·217** ±0·071	**+0·355** ±0·051
Lucerne: uninoculated	**+0·178** ±0·035	*−0·186* *±0·069*	+0·016 ±0·040	—	—	—	**+0·249** ±0·062	*−0·173* *±0·071*	*−0·048* *±0·051*
inoculated	**+0·194** ±0·040	+0·093 ±0·090	+0·105 ±0·045	**+0·250** ±0·057	+0·248 ±0·111	**+0·229** ±0·064	**+0·320** ±0·060	**+0·180** ±0·069	**+0·293** ±0·046
Vetch: uninoculated	**+0·613** ±0·054	*−0·009* *±0·069*	+0·024 ±0·037	*−0·038* *±0·072*	*−0·082* *±0·130*	*−0·021* *±0·082*	—	—	—
inoculated	+0·106 ±0·054	±0·077 ±0·069	*−0·053* *±0·040*	+0·080 ±0·070	+0·129 ±0·130	+0·199 ±0·082	**+0·205** ±0·049	**+0·203** ±0·056	**+0·228** ±0·039
Differential Inhibition by Inoculated and Uninoculated Companion Plants†									
Clover	—	—	—	***−0·343*** *±0·082*	***−0·422*** *±0·184*	*−0·131* *±0·092*	***−0·415*** *±0·071*	***−0·305*** *±0·084*	***−0·215*** *±0·059*
Lucerne	*−0·017* *±0·035*	***−0·279*** *±0·094*	*−0·093* *±0·052*	—	—	—	*−0·077* *±0·068*	***−0·353*** *±0·071*	***−0·334*** *±0·054*
Vetch	**+0·475** ±0·074	*−0·086* *±0·082*	+0·077 ±0·045	*−0·063* *±0·081*	*−0·211* *±0·145*	***−0·206*** *±0·088*	—	—	—

† These differences have not been obtained by simple subtraction of the values in the upper part of the table but have been calculated from the original sets of data in which replication and variances differed.

plants; by clover only when inoculated and not at all or only to a small degree by vetch. Vetch appears on the whole to be the least inhibitory companion plant since it is without effect on lucerne except in slightly depressing dry weight when inoculated, and does not influence rooting or dry weight of clover.

Differential inhibition by the inoculated and uninoculated companion plants of each species is shown in the lower part of Table XIV, and illustrates the points made above.

pH Changes in the Medium

The original medium was buffered at pH 6·8–7·0 but became acid during the growth of the plant. Tests of the agar with indicator show that the acidity develops most strongly in the vicinity of the root, and it is the reaction of this part of the medium which is recorded in Table XV (means of four readings). Nodulated clover and lucerne depress the pH of the medium to about an equal extent; with vetch the depression is slightly less marked. Clover and lucerne are generally the most active plants in both inhibition and in lowering the pH of the medium, but beyond this very general relationship changes in reaction in the medium do not assist in explaining the results, in particular the contrasted effects of inoculated and uninoculated clover on lucerne.

Table XV

pH of Agar in Contact with Roots at End of Experiment: Mean Values of Several Determinations with Indicators

Symbols: Plants—C, clover; L, lucerne; V, vetch.
Bacteria—c, clover strain; l, lucerne strain; v, vetch strain.

Plant.	Bacteria.	pH.	Plant.	Bacteria.	pH.	Plant.	Bacteria.	pH.
C	c	5·4	L	l	5·4	V	v	5·9
CC	c	5·4	LL	l	5·6	VV	v	6·0
C+L	c	5·5	L+C	l	5·7	V+C	v	6·2
C+V	c	5·8	L+V	l	5·5	V+L	v	5·6
C+L	c+l	5·5	L+V	l+v	5·7	V+C	v+c	5·8

Numbers of Bacteria

Previous experience suggests that under the conditions of growth employed the absolute number of bacteria present in an inoculum is of little account in determining the number of nodules formed since a very small original inoculum of bacteria quickly gives rise to a large population in the rhizosphere. Where two different strains of bacteria are present, however, competition or antagonism may lead to the preponderance of one strain and the virtual suppression of the other, as has been shown by Nicol and Thornton (1941) for effective and ineffective strains of pea and clover nodule bacteria in mixed culture in the presence or in the absence of the host plant. Under these conditions nodule formation by the suppressed strain may be reduced

either directly because of the very small number of bacteria present or indirectly by the bacteria of the other strain forming colonies on the exterior of the root-hairs and in so doing preventing the entry of the bacteria of the suppressed strain. With competing strains of different cross-inoculation groups nodule formation may be affected in either of these ways.

An examination of the influence of bacterial numbers was made in part of experiment 3, in which the size of the original inoculum was standardized. Suspensions of each strain were made up in salt solution and adjusted to be of equal turbidity. From these suspensions single-strain inoculations were made with 1 ml. of suspension and 1 ml. of salt solution and double inoculations with 1 ml. of the suspensions of each strain.

Counts were made of the number of viable bacteria present in the liquid part of the medium shortly before harvest by plating on mannitol yeast-water agar. From tubes containing either clover or lucerne or both host plants, three samples were taken and counts made from three parallel plates of each. Where both strains were present colonies were picked at random and identified by agglutination tests. The results are given in Table XVI. It should be pointed out that these estimates are minimal since they refer to the numbers of bacteria free in the solution; a very much larger population of bacteria is present on the root surface and among the root-hairs. The very high bacterial numbers recorded show that the bacterial population is well above the range at which nodule number is affected by variation in the bacterial population (Bhaduri, 1951).

TABLE XVI

Numbers of Clover and Lucerne Nodule Bacteria in Treatments inoculated with One or Both of these Strains

Symbols for plants and bacteria as in Table XV

Treatment.		Numbers as 10^6/ml. Clover bacteria.	Lucerne bacteria.
C,	c	47	—
CC,	c	39	—
L,	l	—	37
LL,	l	—	27
C+L,	c	163	—
C+L,	l	—	23
C+L,	c+l	76	7

S.E. mean per count $\pm 28 \times 10^6$/ml.

Comparing clover bacterial numbers in the presence of clover alone and with both host plants, it would appear that the presence of the lucerne plant has increased the number of clover bacteria considerably, whereas the clover plant has little effect on the number of lucerne bacteria. In mixed cultures of bacteria the presence of a lucerne plant considerably depresses the nodulation of clover and the presence of the clover has a similar effect on the nodulation of a lucerne plant; the effects noted on bacterial numbers therefore bear no

relation to the nodulation of either host. Information on the direct effect of competition is not available in this experiment since no monocultures were inoculated with both strains. Clearly competition between strains of nodule bacteria occur under these conditions but do not provide an explanation of nodule inhibition.

Interaction between Legumes and Non-legumes

The influence of a non-leguminous companion plant on nodulation of clover was examined in two experiments, the first using lettuce and the second flax. No combined nitrogen was added to the medium so that little growth was made by the associated non-legume, least by lettuce because of its smaller seed reserve. In the first experiment nodule counts only were made, in the second roots were counted and dry weights determined; results are shown in Table XVII.

Table XVII

Legume and Non-legume Interaction in Absence of Combined Nitrogen in the Medium

Experiment 1. Interaction between Clover and Lettuce

Companion plant.	Mean log. number of nodules on clover.
None	1·536
1 clover	1·289
1 lettuce	1·439
2 lettuce	1·271
Inhibition due to 1 lettuce	0·097±0·054
Inhibition due to 2 lettuce	0·255±0·049**

Experiment 2. Interaction between Clover and Flax

Companion plant.	Log. nodules (+1).	Log. roots (+1).	Log. dry wt.
None	1·413	1·681	1·778
1 clover	1·165	1·371	1·532
1 flax	1·319	1·615	1·783
Inhibition due to flax	0·094±0·105	0·087±0·096	−0·005±0·059

** $P < 0{\cdot}01$.

With either one or two lettuce plants per culture, nodule formation on clover is inhibited but only to a significant amount with two lettuce plants, the inhibition here being the same as that produced by a single clover plant. With flax as a companion plant fewer roots and nodules are formed on the clover, but in neither case is the inhibition significant. Flax although a larger plant than lettuce would seem, therefore, to be less active in inhibiting the nodule development and the growth of the legume with which it is grown.

The above survey of a wide variety of plant interactions suggests that inhibition in paired cultures is due to some modification of the root medium not connected with the normal nutrition of the plant. The possibility, however, that the mutual shading among the very young seedlings may be responsible

for inhibition is not quite excluded. Although large differences occur in the final sizes of plants taking part in the interactions, in the early stages of growth the competing plants are of the same size. This aspect was investigated in an experiment in which agar slopes were replanted at intervals. A factorial design was adopted with two rates of preplanting using sparsely (S) and abundantly nodulating lines (A) for preplanting and for testing the agar. In one half of the experiment the top of the plant was removed leaving the old roots in the agar, whereas in the other half of the experiment the whole of each plant was removed before replanting the agar slope. The first sowings were made on February 22, the second on April 3, the third on May 15, and the last on June 26, the test plants being harvested on August 29. Each of the 16 treatments were replicated 4 times and in addition a set of 25 control cultures was set up on June 26, on agar which was not preplanted. The results are set out in Table XVIII, showing the difference between the control means and the treatment means, a positive difference indicating inhibition and a negative difference stimulation. Results show that in the experiment as a whole preplanting strongly inhibits nodulation, stimulates root formation, and has little effect on the size of the test plant. As far as

Table XVIII

Experiment on the Effect of Preplanting on the Infection and Growth of Clover

Table of Mean Differences between Control and Treated Plants (Control minus Treated)

Positive values indicate inhibition and negative values stimulation

Symbols: S, sparsely nodulating line of test plant; A, abundantly nodulating line of test plant; PS, preplanted with sparse line; PA, preplanted with abundant line; R, roots of preplanting removed from culture; L, roots of preplanting left in agar; 1, one preplanting; 3, three preplantings.

Preplanting treatment.			Nodules		Roots		Dry wt.	
			S	A	S	A	S	A
1	R	PS	0·146	0·044	−0·256	−0·028	−0·066	0·048
		PA	0·511	−0·126	−0·131	−0·054	0·004	0·072
	L	PS	0·076	0·034	−0·236	−0·138	−0·051	0·063
		PA	0·306	0·303	−0·281	−0·084	−0·136	−0·007
3	R	PS	0·104	0·240	−0·039	0·004	0·038	0·062
		PA	0·190	0·155	−0·093	0·171	0·050	0·108
	L	PS	0·336	0·174	−0·147	0·031	0·026	0·040
		PA	0·403	0·323	−0·134	−0·013	−0·013	0·058
Mean differences			0·179**±0·051		−0·138**±0·042		0·019±0·029	

Analysis.	Nodules.	Roots.	Dry wt.
S *v.* A	0·0708±0·0786	−0·1508±0·0628*	−0·0743±0·0454
1 *v.* 3	−0·1240±0·0786	−0·1233±0·0628*	−0·0553±0·0454
R *v.* L	−0·1315±0·0786	0·0722±0·0628	0·0420±0·0454
PS *v.* PA	−0·0692±0·0786	−0·0239±0·0628	0·0030±0·0454

* $P < 0.05$. ** $P < 0.01$.

nodulation is concerned, therefore, the effects of growing clover plants in association or in succession are the same, whereas for rooting and growth they are opposed. This result lends strong support for the view that nodulation may be inhibited by a root secretion. Further analysis of the results in Table XVIII shows that S and A plants do not differ in their response to preplanting in the number of nodules formed. The relative amount of inhibition is the same, a result in agreement with the data in Tables VI and VII. For rooting, however, S and A plants differ in their response to preplanting, a smaller stimulation occurring on A plants suggesting that at high levels of root production a further limitation to root formation arises.

Differences due to the number of preplantings employed are not quite significant for nodule number ($P = 0{\cdot}1$), although they are in the anticipated direction, three preplantings showing stronger inhibition than a single preplanting. With roots, however, a single preplanting has a greater stimulating effect than three. The residual influence of the root when left in the agar is only noticeable for nodule production where it leads to a somewhat stronger inhibition. This difference approaches a level of significance ($P = 0{\cdot}1$–$0{\cdot}05$) indicating that an excised whole root system may continue to produce some inhibitor after the top has been removed; this effect is small and may be compared with the result reported in the previous paper (Nutman, 1952, p. 94) that no inhibition followed the addition of small numbers of cut off nodules or root tips to a culture.

The character of the plant is without effect on inhibition or stimulation and is thus again in agreement with the results summarized in Tables VI and VII. No differential effects for dry weight were observable for any of the four factors at two levels, and none of the six interactions between the factors for nodule roots or dry weights were significant.

Discussion

In the above study of the interaction between species and varieties of plant sharing the same root space, a high degree of consistency is shown in the general effects and responses.

All plants interact to a greater or less degree when grown together, the degree of inhibition of nodule formation bearing no relation to the size of the plants concerned even when size has been varied as widely as experimental procedures will permit. This complete independence of size disposes of a simple hypothesis based on competition and favours the proposed alternative, i.e. that inhibition of nodule formation is due to a substance secreted by the roots.

Among the very wide range of interactions examined in clover, evidence for differences in inhibition was obtained only for plants which differed in the stage of seedling development at which nodules were first formed, selections of early nodulating plants inhibiting nodule formation on another plant more strongly than plants selected for late nodulation (Table IX and Fig. 5). This character, viz. earliness of nodulation, has been attributed by Thornton (1929)

and Ludwig and Allison (1935) to the production of a nodule-stimulating substance and this hypothesis needs examination in the light of the experiments here presented. In the case of lucerne it has been shown that the presence of a second lucerne plant encourages nodulation in conformity with the hypothesis. Moreover, the substitution of a different companion plant (clover or vetch) for the lucerne has a similar effect on the initiation of nodules on lucerne so that it would appear that the stimulatory material is not specific to lucerne.

The position with regard to clover is quite different, for in this case the presence of a second clover plant does not advance nodulation, but tends to delay it, whereas the presence of a lucerne or vetch plant again tends to induce earlier nodulation. It may, therefore, be possible that lucerne produces a stimulatory substance which affects not only itself but also clover. The position with regard to vetch is uncertain.

To elucidate the data presented in this paper, however, the part played by a possibly stimulatory substance must be regarded as of secondary importance. The main effects shown throughout point to the presence of an inhibitory substance. Thus (1) the inhibitory effect in clover is proportional to the number of plants in a given volume of medium (Table III); (2) in cultures with varying number of plants (1–3) the nodule numbers are the same provided that each plant occupies an equal volume of medium (Tables II and III); (3) by successive replanting of the same volume of medium the degree of inhibition increases, being greater after three plantings than after one (Table XVIII).

To investigate the specificity of the inhibition three legumes have been used, viz. clover, lucerne, and vetch, which have been grown as single plants and in pairs consisting of plants of the same species and of members of two of the species. In every case, but to varying degrees, the presence of a second plant reduces the nodulation of its companion plant below that of a single plant. Two aspects of the inhibition must be considered: (1) inhibition *by* the plant of a given species, and (2) inhibition *on* a plant of a given species. In the former, two cases must be further distinguished: (*a*) inhibition by plants inoculated with their appropriate bacteria, and (*b*) inhibition by uninoculated plants.

Inoculated clover plants uniformly inhibit the nodulation of another clover, lucerne, or vetch plant, the inhibition being least in clover and greatest in vetch. Uninoculated clover, on the other hand, has little effect on lucerne or vetch, but by using a genetically resistant line of clover the effect of an un-nodulated clover plant on a normally inoculated clover plant was tested and was found to be strongly inhibitory (Table XI). So far as lucerne or vetch are concerned the effect of un-nodulated plants could not be tested against a nodulated plant of the same species as no resistant lines of these plants are available.

On clover and vetch un-nodulated lucerne inhibits, again least in clover and most in vetch, and with inoculated lucerne the effects are more strongly

inhibitory, again being least on clover and greatest on vetch. In the case of uninoculated vetch a large inhibitory effect is seen on clover and no effect appears on lucerne, whereas nodulated vetch has little effect on clover or lucerne and the greatest effect on itself. In general the nodulated plants have a larger effect on their companion plants than un-nodulated plants, but the interspecific effects cannot be correlated in any obvious way with the characteristics of the plants. Inhibiting effects are also obtained with plants other than legumes, as was shown by growing flax and lettuce as companion plants to clover, the nodulation of which was reduced by either species.

In previous papers in this series the hypothesis has been put forward that the capacity for nodulation in clover is intimately associated with capacity for lateral root formation (Nutman, 1948, 1949*b*, 1952). In support of this hypothesis it was shown experimentally that the apical meristems of the roots or of the nodules produce inhibitory effects both on lateral formation and on nodulation and that the removal of either the root or nodule meristem increased the rate of nodulation. Data on root production has been collected during the course of this work and in general it may be said that the inhibitory effect on nodule formation is associated with reduced number of lateral roots. Two exceptions to this rule have been encountered, viz. (1) lucerne and vetch, whether inoculated or uninoculated, inhibit nodulation on clover without affecting the number of lateral roots produced; (2) successive resowings of clover on the same medium leads to a progressive inhibition of nodulation accompanied by a *stimulation* of lateral roots. All interspecific effects on clover, including that of non-leguminous companion plants, conform with the general statement that reduction of nodulation is accompanied by reduction in lateral root formation. To account for these aberrant results will require further work; at the present stage of the investigation it is only possible to speculate on their interpretation. It might be assumed, for example, that differences in the response of roots and nodules may be due to different levels of inhibitor at the sites of initiation of these organs or to differences in susceptibility to a given level of inhibitor. That contrasted effects may be obtained by varying the concentration of an inhibitor has been generally accepted in the case of the action of auxin on roots (Amlong, 1936; Feidler, 1936 and others) where very low concentrations of the order of 10^{-9}/mols/litre accelerate root growth whereas higher concentrations inhibit it. In experiments with clover resown in the same medium nodule production is reduced and root production stimulated, which would indicate that nodules are more sensitive to inhibition than the root, a suggestion strengthened by the fact that repeated resowing of the medium leads to a *lessened* stimulation of root development accompanied by increased inhibition of the nodules.

Alternatively it might be postulated that there are two inhibitory substances having independent effects on roots and nodules, or, as suggested in the last paper, that the meristematic foci situated nearest the absorptive zone of the roots are most affected by inhibition. In the absence of experimental

confirmation such speculations carry little weight and it will be the object of further work to explain these aberrant results and also to determine whether the inhibitory effect due to associated growth is in any way connected with the inhibitory activity of the meristems of the effective nodule or lateral root discussed in previous papers of this series.

Summary

1. The number of nodules formed on a clover plant grown on an agar slope in a test-tube is related to the volume of the medium and to the number of plants growing within the same culture tube in such a way that the number of nodules per unit volume of medium per plant tends to a constant value.

2. Nodule inhibition on clover has been examined using associations of the following selected types of plant: effectively responding, ineffectively responding, resistant, sparsely nodulating, abundantly nodulating, plants which form nodules early in development, and plants which form nodules late in development.

3. The degree of inhibition experimentally obtained was quite unrelated to the size of the interacting plants, which varied considerably between plant categories, but was correlated with earliness or lateness in primary nodule development. Plants which formed nodules early in development inhibited nodulation on companion plants more strongly than late nodulating plants.

4. Flax and lettuce reduced nodulation on clover plants with which they were grown but to a much less extent than companion clover plants.

A study of the interaction between clover, lucerne, and vetch was made using the same method of culture, with the following results:

5. Plants of the same species always formed fewer nodules per plant when grown in association than when grown singly.

6. Nodulation on clover was reduced by the presence of either inoculated or uninoculated lucerne or vetch, but nodulation on lucerne was depressed only by inoculated clover, and on vetch by inoculated and uninoculated lucerne and inoculated clover and not by uninoculated clover (Table XIV).

7. On lucerne the time at which the first nodules were initiated was advanced in paired cultures with either clover, lucerne, or vetch as companion plants. Nodulation time on clover or vetch was not significantly affected by association.

8. Interspecific interactions were not related to the number of nodule bacteria of the appropriate strain present in the root surroundings nor to changes in pH in the medium.

9. Inhibition of nodule formation on clover was obtained in cultures which had been preplanted with clover and the earlier plantings removed.

10. In general, effects of associated growth on nodule formation were highly correlated with the number of lateral roots formed and dry weight increase in the plants.

11. The results are interpreted in terms of the secretion of nodule inhibiting substances from the roots.

Acknowledgements

The author's sincere thanks are due to Prof. F. G. Gregory, F.R.S., and also to Dr. H. G. Thornton, F.R.S., for stimulating and helpful advice and criticism during the carrying out of the work and in the preparation of the manuscript; to Mr. M. J. R. Healy and Mr. J. K. R. Wood for guidance and help in the statistical analyses; to Miss Hilary Purchase for making the bacterial counts, and to Miss Mabel Dunkley, Miss Joan Crawley, and Mr. Michael Shaw for technical assistance.

Literature Cited

Amlong, H. U., 1936: Der Einfluß des Wuchsstoffes auf die Wanddehnbarkeit der Vicia-Faba-Wurzel. Ber. d. bot. Ges., liv. 275.

Bhaduri, S. N., 1951: Influence of the Numbers of Rhizobium supplied on the Subsequent Nodulation of the Legume Host Plant. Ann. Bot., n.s., xv. 209.

Fiedler, H., 1936: Entwicklungs- und reiz-physiologische Untersuchungen an Kulturen isolierter Wurzelspitzen. Zent. f. Bot., xxx. 385.

Fred, E. B., Baldwin, I. L., and McCoy, E. F., 1932: Root Nodule Bacteria and Leguminous Plants. Univ. Wisconsin Studies in Sci., v. 343.

Ludwig, C. A., and Allison, F. E., 1935: Some factors affecting Nodule Formation on Seedlings of Leguminous Plants. Journ. Amer. Soc. Agron., xxvii. 895.

Nicol, H., and Thornton, H. G., 1941: Competition between Related Strains of Nodule Bacteria and its Influence on Infection of the Legume Host. Proc. Roy. Soc., London, B, cxxx. 32.

Nutman, P. S., 1945: A Factor in Clover Nodule Formation associated with the Volume of the Medium occupied by the Roots. Nature, clvi. 20.

—— 1948: Physiological Studies on Nodule Formation. I. The Relation between Nodulation and Lateral Root Formation in Red Clover. Ann. Bot., n.s., xii. 81.

—— 1949*a*: Nuclear and Cytoplasmic Inheritance of Resistance to Infection by Nodule Bacteria in Red Clover. Heredity, iii. 263.

—— 1949*b*: Physiological Studies on Nodule Formation. II. The Influence of Delayed Inoculation on the Rate of Nodulation in Red Clover. Ann. Bot., n.s., xiii. 261.

—— 1952: Studies on the Physiology of Nodule Formation. III. Experiments on the Excision of Root-tips and Nodules. Ibid., xvi. 79.

Ridgway, R., 1912: Color standards and Color Nomenclature, Washington, D.C., U.S.A.

Thornton, H. G., 1929: The Influence of the Number of Nodule Bacteria applied to the Seed upon Nodule Formation in Legumes. Journ. Agric. Sci., xix. 373.

A new Species of *Stylopage* capturing Amoebae in Dung

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With one Figure in the Text

ABSTRACT

A species of *Stylopage* with branched conidiophores and broad ellipsoidal spores is described for the first time. This fungus captures amoebae by means of its sticky hyphae, absorbing the contents of the animals with slender filamentous haustoria.

THIS fungus occurred in a load of horse-dung from a stable near Kingston-on-Thames, delivered for use as garden manure. Small portions of the dung were placed on plates of sterile maize-meal agar, and the mixture of fungi that grew out over the surface of the medium was kept under observation. About a fortnight after the plate had been inoculated, small amoebae that had wandered out of the inoculum were being captured in large numbers by a fungus that was clearly a member of the Zoopagaceae.

The mycelium consisted of straight, non-septate hyphae, 1–2 μ in diameter, spreading over the surface of the medium and branching frequently. Amoebae that touched the mycelium were captured, apparently by adhesion to the hyphae. Shortly after an amoeba had been captured, a slender branch from the hypha grew out through the ectoplasm of the animal and penetrated into the endoplasm; this haustorial branch was usually about 1 μ in diameter and was often forked (Fig. 1*a*, *b*). Other branches grew out, so that ultimately the endoplasm of the captured animal became filled with delicate filaments, all arising from the same point on the mycelium and spreading in a bush-like manner within the endoplasm of the prey, which was by this time moribund. Eventually the entire contents of the animal were absorbed by the fungus, leaving only the shrivelled remains of the ectoplasm still attached to the mycelium.

Spore formation began after an initial period of feeding. From the mycelium erect fertile hyphae rose to a height of about 150 μ, each bearing at its apex a conidium. Usually a second conidium was produced by continued growth of a branch of the fertile hypha. This proliferation was usually repeated several times, so that up to eight conidia were formed by sympodial branching of the conidiophore (Fig. 1*d*). The conidia were rectangular-ellipsoidal, hyaline and one-celled, 12–21 μ long and 6–10 μ wide (Fig. 1*e*). They

[Annals of Botany, N.S. Vol. XVII, No. 65, January, 1953.]

germinated freely in old cultures, each usually producing a secondary conidiophore with a secondary conidium.

Sexual reproduction was not observed, although cultures were watched until the fungus had apparently ceased to grow.

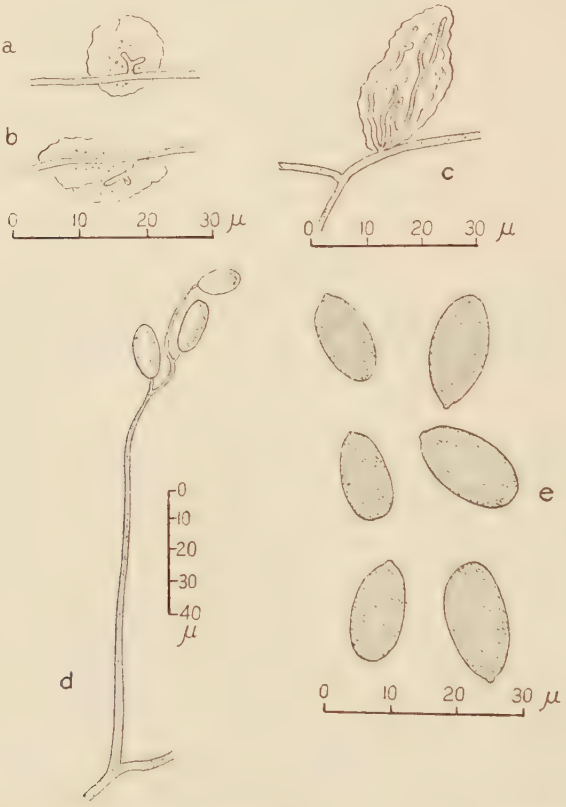

FIG. 1. *Stylopage cymosa* n.sp. *a*, *b*. Young haustorial branches developing in captured amoeba. *c*. Fully developed haustorial system. *d*. Conidiophore, bearing three conidia. *e*. Conidia.

The non-septate mycelium, the method of capturing amoebae, and, particularly, the long fertile hyphae bearing non-septate conidia indicate that this fungus is a species of *Stylopage* Drechsler. The conidia are not unlike those of *S. araea* Drechsler (1935), but are distinctly broader and blunter ended; in *S. araea*, moreover, the conidiophore bears only a single spore. Proliferation of the fertile hyphae is seen in *S. lepte* Drechsler (1935), but here the conidia are very slender; it is also shown by *S. leiohypha* Drechsler (1936), but this species has much larger spores and captures nematodes. The fungus here described is therefore proposed as a new species with the binomial *Stylopage cymosa*, the epithet being chosen on account of the marked sympodial branching of the conidiophore.

Stylopage cymosa sp. nov. Mycelium effusum, parce ramosum, hyphis continuis, hyalinis, 1–2 μ crassis, ad amoebas inhaerentibus, et haustoria filiformia intus evolventibus. Hyphae fertiles erectae, circa 150 μ longae, 1–8 conidia continua efficientes. Conidia hyalina, ovata, 12–21 μ longa, 6–10 μ crassa. Zygospora ignota.

Amoebas capiens consumensque hab. in fimo equi prope Kingston-on-Thames, Surrey, March 1951.

Type material of *Stylopage cymosa* has been deposited in the Herbarium of the Royal Botanic Gardens, Kew, and in the Herbarium of the Commonwealth Mycological Institute, Kew.

All drawings and measurements used in this paper have been made from living material, unless otherwise stated.

I should like to record my thanks to Miss S. M. Dixon for her kind assistance in preparing the Latin diagnosis.

Literature Cited

Drechsler, C., 1935: Some Conidial Phycomycetes destructive to Terricolous Amoebae. Mycologia, xxvii. 6–40.

—— 1936: A New Species of *Stylopage* preying on Nematodes. Ibid., xxviii. 241–6.

The Diatom Genus *Tabellaria*

II. Taxonomy and Morphology of the Plankton Varieties

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With seven Figures in the Text

ABSTRACT

Plankton samples of *Tabellaria* from all the lakes of the English Lake District have been examined and evidence of morphologically distinct populations has been found in samples from (*a*) a single lake at one time, (*b*) different lakes, and (*c*) a single lake at different times. Clone cultures have been used to study the nature of these differences. Fifteen characters have been found to show quantitative or qualitative variation in plankton populations of *Tabellaria*, and the morphological diversity of different populations is largely due to different combinations of these characters. All known plankton populations of *Tabellaria* are referred to the species *T. flocculosa*.

I. INTRODUCTION

IN the first paper of this series the author has shown that the specific limits of *Tabellaria flocculosa* must be widened to include taxa which were formerly placed in the species *T. fenestrata*. The emended species *T. flocculosa* was shown to include the three plankton varieties *T. flocculosa* var. *asterionelloides* (Grun. in V.H.) Knud., *T. flocculosa* var. *geniculata* (A. Cleve) Knud., and *T. flocculosa* var. *pelagica* Holmboe. Two other plankton varieties (*T. fenestrata* var. *willei* Huitfeldt-Kaas and *T. fenestrata* var. *lacustris* Meister) also form part of the emended species *T. flocculosa*. If any or all of these five varieties are to be retained it is necessary to demonstrate that they are (*a*) distinguishable from each other and (*b*) distinguishable from *T. flocculosa* var. *flocculosa*. This paper deals with the morphology of these varieties and the evidence from this of their taxonomic status. Later papers will deal with their ecological and geographical distribution, and with their relationship to *T. flocculosa* var. *flocculosa*.

Apart from *T. flocculosa* var. *geniculata*, whose claim to varietal rank has seldom been questioned, opinions vary on the status and synonymy of the other plankton varieties, as recourse to the works of Hustedt (1931), Huber-Pestalozzi (1942), Teiling (1944), and Thunmark (1945) will show. Reference to the microphotographs in W. and G. S. West's (1905) paper on Scottish plankton will also reveal to what diverse types of colony the name *T. fenestrata* var. *asterionelloides* has been attached.

[Annals of Botany, N.S. Vol. XVII, No. 65, January, 1953.]

This account of the taxonomy and morphology of the plankton varieties of *Tabellaria* falls into four main sections:

1. Characters of the five varieties.
2. Morphological variation in plankton *Tabellaria* from natural waters. The following kinds of variation are studied:
   (*a*) variation in a single plankton sample;
   (*b*) variation from lake to lake;
   (*c*) seasonal variation;
   (*d*) long-term variation.
3. The taxonomic value of various characters in delimiting plankton varieties as shown by a study of variation in nature and in culture. The following characters are examined:
   (*a*) colony morphology;
   (*b*) frustule symmetry;
   (*c*) apical/pervalvar axis ratio and the number of septa per cell;
   (*d*) length range.
4. Discussion and conclusions.

II. Characteristics of the Previously Published Plankton Varieties of *Tabellaria*

1. *T. flocculosa* var. *asterionelloides* (Grun. in V.H.) Knud.
   T. fenestrata var. *asterionelloides* Grunow in Van Heurck, Syn. Diat. Belg. pl. 52, fig. 9 (1881).
   T. flocculosa var. *asterionelloides* (Grun. in V.H.) Knud., Ann. Bot. Lond. (1952).

As stated in the first paper of this series, a neotype of this variety has been made on account of the apparent absence of a holotype. This comes from the type locality, Hästefjord, and is a slide in the British Museum (Natural History) (B.M. 12815, Cleve and Möller 75). The characters of this neotype may be summarized as follows:

Colony morphology: about 70 per cent. of the colonies are star-shaped (Fig. 1, B), the rest being zigzag colonies (Fig. 1, A) with a similar angle between their cells as in those of the first kind. The star-shaped appearance is due to the fact that the colony is a short flattened helix, a complete turn of which contains from five to eight rays (each ray consists of one cell or two unseparated daughter-cells).

Cell morphology: apical axis from 9·5 to 16·3 times greater than the pervalvar axis. The cells taper slightly towards the outside of star-shaped colonies.

Length range: 51–72 μ.

Septa: 2–5 per cell. Rudimentary septa are very poorly developed. In the star-shaped colonies, cells with an uneven number of septa tend to have more septa at the inner than at the outer pole. Thus out of 29 such cells, 20 had more septa at the inner pole.

The widely held opinion that this plankton diatom and *T. fenestrata* are interconvertible has been criticized in a previous paper (Knudson, 1952). The issue that remains is whether the predominantly stellate plankton forms of *T. flocculosa* are varietally distinguishable from the attached ones, i.e. whether or not *T. flocculosa* var. *asterionelloides* is distinct from *T. flocculosa* var. *flocculosa*. This problem will be examined in a later paper.

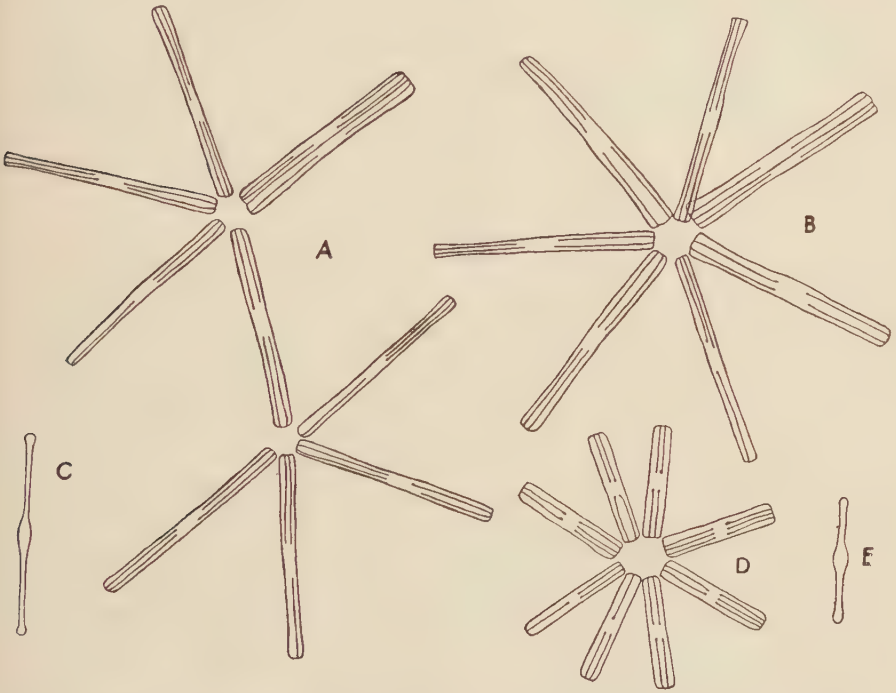

FIG. 1. A–C. *Tabellaria flocculosa* var. *asterionelloides* (Grun. in V.H.) Knud. A, B, colony morphology; C, valve contour. Drawn from the neotype (Cleve and Möller. No. 75; B.M. 12815). D, E. *Tabellaria fenestrata* var. *lacustris* Meister. D, colony morphology; E, valve contour. (After Meister.) (A–C, ×410; D, E, ×295.)

2. *T. fenestrata* var. *lacustris* Meister in Beiträge zur Kryptogamenflora der Schweiz, Band IV, Heft 1, Bern, 1912, pp. 55–56 and pl. IV, figs. 6–7.

Meister gives the following description of *T. fenestrata* var. *lacustris*: 'Valve length 38–95 μ, breadth in the middle 6–8 μ, at the ends 5–7 μ, between the swellings 3–4 μ. The cells are sometimes united to form star-configurations (forma *asterionelloides* Grun.), sometimes to form zigzags. The number of septa is predominantly two on each side. Not uncommon in the plankton of lakes. Abundant in Zürichsee since 1896.'

Meister's drawing (reproduced in Fig. 1, D) shows a colony of valve length 50 μ from Zürichsee. The eight cells of which it is composed do not taper and they are arranged in one plane. There are from three to six septa per cell.

The author does not give his reasons for making this new taxon and relegating Grunow's var. *asterionelloides* to the status of a forma. He seems to have been prompted by a dislike of the name var. *asterionelloides* for a population composed of zigzag as well as star-shaped colonies! His procedure was illegal and in order to retain the taxon it would be necessary to show that (1) *T. fenestrata* var. *lacustris* differs from *T. flocculosa* var. *asterionelloides* and (2) that the characters by which it differs are of taxonomic importance.

Comparison of the above description with that of *T. flocculosa* var. *asterionelloides* reveals that the following characters differ: (*a*) number of septa per cell; (*b*) tapering of cells; (*c*) number of rays in one turn of the helix. The taxonomic significance of these characters will be discussed in a later section.

3. *T. fenestrata* var. *willei* Huitfeldt-Kaas in Planktonundersøgelser i norske vande. Christiania (1906), p. 42.

The following is Huitfeldt-Kaas' description (translated from the German): 'Length of the cell *c.* 30 μ, breadth *c.* 4·7 μ. This variety with its star-arrangement of the cells is very much like *T. fenestrata* var. *asterionelloides* but differs from the latter in its much smaller size. In those two—incidentally the only—lakes where I have found this variety, namely Orrevandet and Söilandsvandet on Jaederen, it occurred together in quite considerable numbers with *T. fenestrata* var. *asterionelloides* which was also abundant. Both forms vary somewhat in size. Intermediate forms could not be observed, which therefore indicates that they were not merely different stages in development of the same form. In order to demonstrate the difference between the two forms mentioned above I have illustrated var. *willei* next to var. *asterionelloides*, both from Orrevandet (3.10.'98) and both of the usual size found there (somewhat diagrammatic). The measurements of var. *asterionelloides* are: length 90 μ and breadth 6 μ. The variety is named after Prof. N. Wille.' The author's drawings are reproduced in Fig. 2, A and B.

Huitfeldt-Kaas says 'intermediate forms could not be observed, which indicates that they were not merely different stages in development of the same form', but his conclusion is subject to considerable doubt (see p. 142). Populations of diatoms of the same strain may contain individuals which, because of age and other differences, fall into two distinct morphological groups, and much additional information is required before the two groups can be proved to be distinct varieties. If the '*T. fenestrata* var. *willei*' and the '*T. fenestrata* var. *asterionelloides*' of Huitfeldt-Kaas' sample should be found to be members of a single morphological series, then we should have the very interesting phenomenon that a reduction of approximately 4:1 in the valve length produces no significant alteration in the length of the pervalvar axis.

4. *T. flocculosa* var. *pelagica* Holmboe in Undersøgelser over Norske Ferskvandsdiatoméer. I Diatoméer fra indsjøer i det sydlige Norge. Arch. Math. Naturvid. 22 (1), p. 27 (1899).

This diatom was first noticed in Gjendin by Huitfeldt-Kaas, but the earliest

description, unaccompanied by an illustration, is given by Holmboe (1899): 'cells joined in star-formation, analogous with *T. fenestrata* var. *asterionelloides*'. Holmboe describes the type locality, Gjendin, as 'a high Norwegian mountain lake'.

Later Huitfeldt-Kaas (1906, p. 62) remarks: 'I have only found this new variety, described by Holmboe (1900) in the four adjacent alpine lakes,

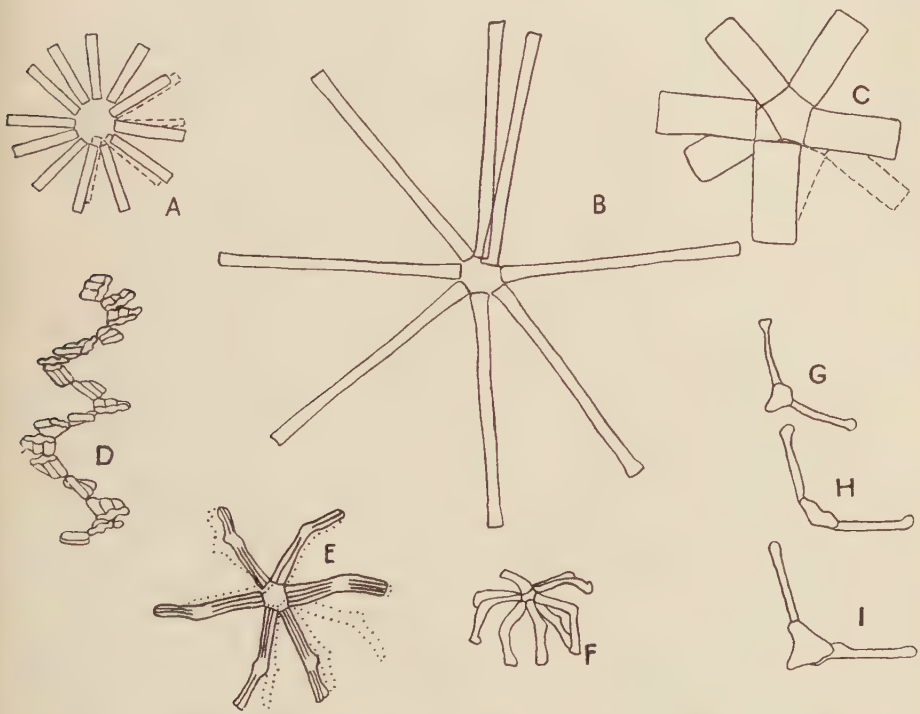

FIG. 2, A. *Tabellaria fenestrata* var. *willei* H.-Kaas, colony morphology. B, *T. flocculosa* var. *asterionelloides*, colony morphology. C, *T. flocculosa* var. *pelagica* Holmboe, colony morphology. D, *T. flocculosa* var. *teilingii* Knud., colony morphology. E–I, *T. flocculosa* var. *geniculata*. (A. Cleve) Knud. E, F, colony morphology. G–I, intercalary bands and septa in valve view. (A–C after Huitfeldt-Kaas; D after Teiling; E–I after A. Cleve.) (A–C, ×400; D. ×166; E–I, unknown.)

Besvand, Gjendin, upper and lower Sjødalsvand, also in smaller numbers in Mjösen, whither this variety was probably transported from the lakes mentioned above by way of the R. Laagen. To Holmboe's description of this variety I will add as a particularly characteristic feature that the star-shaped colonies when fully developed form several, sometimes four to five, spirally-wound coils on account of which the colony, seen from the side, reminds one very much of a screw-thread. The length of the cell varies not inconsiderably, generally I found the length *c.* 40 μ. The breadth varies very much even in the same colony.' Huitfeldt-Kaas' drawing of *T. flocculosa* var. *pelagica* is reproduced in Fig. 2, C. The original does not show septa.

Many authors (e.g. Hustedt, 1931) have regarded *T. flocculosa* var. *pelagica* as a synonym of *T. flocculosa* var. *asterionelloides*. Comparison of Huitfeldt-Kaas' three figures labelled *T. fenestrata* var. *asterionelloides*, *T. fenestrata* var. *willei*, and *T. flocculosa* var. *pelagica* will show the difficulties in accepting such a view. His drawings of *T. fenestrata* var. *willei* and *T. flocculosa* var. *pelagica* show colonies composed of cells of very similar valve length, yet they are strikingly different in appearance. It is very difficult to believe that both *T. fenestrata* var. *willei* and *T. flocculosa* var. *pelagica* form part of the same morphological series as *T. flocculosa* var. *asterionelloides*. Since the neotype of *T. flocculosa* var. *asterionelloides* does not contain any cells shorter than 51 μ, synonyms cannot be established without recourse to evidence from other natural populations and cultures.

The concept of the taxon *T. flocculosa* var. *pelagica* Holmboe has been further modified by Teiling (1942, p. 68). Teiling does not state whether he has seen Holmboe's type material or whether he has collected from the type locality, but because his material (from some Swedish lakes) contains corkscrew-like colonies composed of *twisted* cells, he assumes that the latter character was overlooked by Holmboe and Huitfeldt-Kaas and consequently re-describes *T. flocculosa* var. *pelagica* in terms of his own material. He says: 'the twist is arranged perpendicular to the axis of the screw so that the outside edges are placed like the steps of a winding staircase, whilst the inner edges follow the spiral line' (Fig. 2, D).

Dr. Teiling has very kindly sent me a sample from Täftlången (provins Östergötland) containing the variety which he has called (1947, p. 230) *T. flocculosa* var. *pelagica* Holmboe emend. Teiling. The material is preserved in formalin and the colonies have fragmented into portions corresponding roughly to one turn of the original corkscrew-like structure. The morphology of these fragmented colonies is illustrated in Fig. 3. Thunmark (1945, pp. 178–81, figs. 42 and 43) accepts Teiling's views on this variety. His paper includes microphotographs of *T. flocculosa* var. *pelagica* emend. Teiling, and a list of Swedish lakes where it has been found.

Neither Thunmark nor Teiling gives any proof that his Swedish material is identical with that which Holmboe and Huitfeldt-Kaas collected and described from the type-locality in Norway (Gjendin). This may be so, but evidence contrary to this view should be stated. Teiling (1944, p. 42) mentions that *T. flocculosa* var. *pelagica* is characteristic of oligotrophic waters, whereas *T. fenestrata* (the star-form is included under this species) is 'overwhelmingly eutrophic'. Thunmark (loc. cit.) also shows that the two varieties are distinct ecologically. It is therefore remarkable to find that Holmboe (loc. cit.) records both *T. fenestrata* var. *asterionelloides* and *T. flocculosa* var. *pelagica* from Gjendin! Moreover, Huitfeldt-Kaas (1906) records the presence of both varieties in Mjösen. One explanation of this is that the '*T. fenestrata* var. *asterionelloides*' in the Gjendin samples consisted of long specimens of *T. flocculosa* var. *pelagica* (cf. Fig. 3, A). Another explanation of the discrepancy is that Holmboe's and Huitfeldt-Kaas' material is different from

Teiling's and Thunmark's, and that the colonies from Gjendin and adjacent lakes closely resemble those described from Coniston Water (p. 142). Only the discovery of Holmboe's type material can solve this problem. Present-day collections from Gjendin, though extremely valuable, would not necessarily be conclusive (see p. 146).

It is therefore apparent that no evidence has yet been brought forward to substantiate the view that Holmboe's type material of *T. flocculosa* var. *pelagica* had frustules with a twist. Until the question is finally settled, it seems

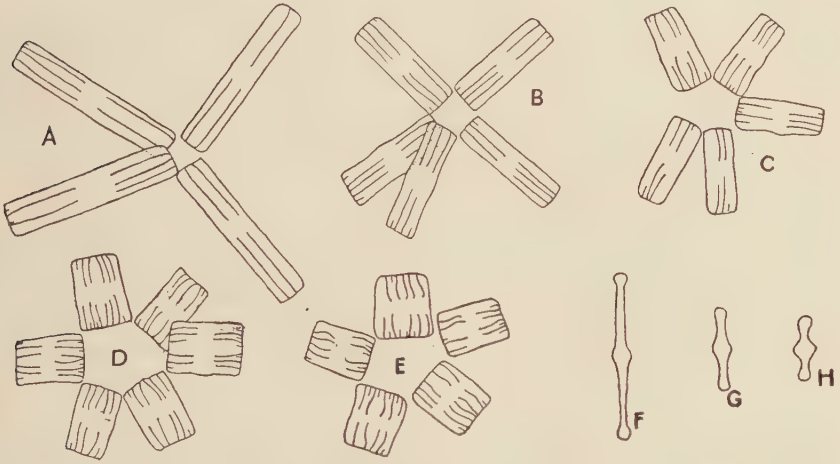

FIG. 3. A–H. *Tabellaria flocculosa* var. *teilingii* Knud. A–E, Fragmented colonies with cells in girdle view. F–H, valve contours. (×410.)

desirable to treat the two concepts separately and to give Teiling's material a separate name. This obviates the use of the cumbersome title *T. flocculosa* var. *pelagica* Holmboe emend. Teiling. I therefore propose the new taxon *T. flocculosa* var. *teilingii*. The following diagnosis is based on Teiling's descriptions (1942, p. 68 and fig. 6; 1944, p. 42 and fig. 72) and his material from Täftlången (provins Östergötland).

T. flocculosa var. *teilingii* nov. var. Coloniae cocleatae, rarissime zigzag ad instar existentes. Frustula torta, axe apicali qui est proportio comparate parva axis pervalvaris. Valvae 17 μ–52 μ longae.

Typus in Herb. Mus. Brit., coll. Diat. No. B.M. 36266, paratypus No. B.M. 36267; ab. 'Täftlången', provincia 'Östergötland', Suecia.

T. flocculosa var. *teilingii* nov. var. Colonies screw-like, very rarely becoming zigzag in form. Frustules twisted, with a comparatively low apical/pervalvar axis ratio. Valves 17 μ–52 μ long.

Holotype in British Museum (Natural History) coll. Diat. No. B.M. 36266, paratype No. B.M. 36267; from Täftlången, provins Östergötland, Sweden.

In the remainder of this paper, therefore, '*T. flocculosa* var. *pelagica*' refers

to specimens agreeing with Holmboe's and Huitfeldt-Kaas' descriptions of material from Gjendin and adjacent lakes, and '*T. flocculosa* var. *teilingii*' to specimens having a corkscrew colony morphology and twisted frustules as in Teiling's material from Täftlången.

5. *T. flocculosa* var. *geniculata* (A. Cleve) Knud.

T. fenestrata var. *geniculata* A. Cleve in Öfvers VetenskAkad. Förh., Stockh. 56 pp. 825–835 (1899).

T. flocculosa var. *geniculata* (A. Cleve) Knud. in Ann. Bot. Lond. (1952).

A. Cleve's original description runs as follows: 'Frustules with a geniculate, lateral flexion in the middle, both ends of the diatom thus making an angle of about 120°. Individuals disposed in radiate aggregates, sometimes forming two superposed spiral turns. Length of the valve 0·1 mm., breadth of the valve when not dilated, 0·004 mm. The central part of the valve has a most variable shape. In the best marked forms it is prolonged on the outerside into a kind of crest.' A. Cleve's illustrations are reproduced in Fig. 2, E–I.

Dr. A. Cleve-Euler has kindly sent me a slide of type material from Virijaure (Lule Lappmark). This plankton sample (5/7/96) closely resembles that from Törnetrask (p. 140) and is equally difficult to interpret. The '*T. fenestrata* var. *asterionelloides*' recorded by her from Virijaure is based on specimens like those of Fig. 5, D–M. The structure of short cells of *T. flocculosa* var. *geniculata* is therefore uncertain.

Table I summarizes the main features of these plankton varieties according to the descriptions and drawings of their original authors. It also suggests which are most likely to prove synonymous.

TABLE I

	Taxon.	Description.	Notes.
1.	*T. flocculosa* var. *asterionelloides* (Grun. in V.H.) Knud.	Colonies predominantly star-shaped. Cells with high apical/pervalvar axis ratio.	
2.	*T. fenestrata* var. *lacustris* Meister.	Colonies star-shaped or zigzag. Cells with high apical/pervalvar axis ratio.	Probably synonymous with 1.
3.	*T. fenestrata* var. *willei* Huitfeldt-Kaas.	Colonies star-shaped. Cells with high apical/pervalvar axis ratio.	Probably synonymous with 1.
4.	*T. flocculosa* var. *pelagica* Holmboe.	Colonies star-shaped or corkscrew-like. Cells with low apical/pervalvar axis ratio.	
5.	*T. flocculosa* var. *teilingii* nov. var. (≡ *T. flocculosa* var. *pelagica* Holmboe emend. Teiling).	Colonies corkscrew-like. Cells with low apical/pervalvar axis ratio. Cells twisted.	? synonymous with 4.
6.	*T. flocculosa* var. *geniculata* (A. Cleve) Knud.	Colonies parachute-like. Cells geniculate.	

It is apparent that no decision about the relationships and separability of these taxa can be reached without recourse to other samples of *Tabellaria*. Only then can we assess the significance of such characters as colony morphology, twisting, and the relationship between the apical and pervalvar axes which have been used as diagnostic features of these plankton varieties.

III. Morphological Variation in Plankton Tabellaria

Variation in a single plankton sample

We have already seen (p. 132) that the neotype of *T. flocculosa* var. *asterionelloides* consists of a mixture of star-shaped and zigzag colonies. Three reasons can be given for believing that the population is genetically homogeneous. These are: (*a*) the length frequency of cells from both types of colony is the same; (*b*) the angle between the cells is the same in both types of colony; (*c*) in all other respects the constituent cells of these colonies seem to be similar.

Sometimes, however, natural populations of plankton *Tabellaria* show signs of being genetically heterogeneous. Two examples of this will now be considered. The first is a plankton sample from Ullswater (14/9/1949). This sample appeared to be a mixture of at least three populations whose characters are summarized in Table II.

Table II

Colony morphology.	Cell morphology.	Length range.	Figure.
(1) Star-shaped with 5–12 rays per turn of the helix; occasional colonies show transitions to the zigzag condition and in colonies of both types adjacent cells lie at an acute angle to each other.	Cells strongly twisted.	49–55 μ	4, A
(2) Star-shaped with 6–10 rays per turn of the helix; occasional colonies show transitions to the zigzag condition and in colonies of both types adjacent cells lie at an acute angle to each other.	Cells untwisted.	40–51 μ	4, B
(3) Zigzag with majority of cells at right angles to each other; occasional colonies end in four-celled stars. Four-rayed stars (i.e. with a right angle between adjacent cells) may also be found alone.	Cells slightly twisted.	43–67 μ	4, C

Examples of these three types are illustrated in Fig. 4. It should be noted that the length ranges of the twisted and untwisted kinds of star-shaped colonies (i.e. (1) and (2)) overlap slightly. The question therefore arises, Are populations (1) and (2) members of a single morphological series? Decrease in frustule complexity with decrease in valve-length has been recorded in other diatoms, so it is not improbable that the twist becomes lost in frustules below about 50 μ in valve-length and that populations (1) and (2) are members of a single morphological series.

If this sample from Ullswater had been the only one available from that lake it is difficult to see how a conclusion could have been reached, but in an Ullswater sample collected 17/10/1951, twisted star-shaped colonies of valve length 38 μ were discovered. This, I believe, supports the hypothesis that populations (1) and (2) in the 1949 sample were two distinct 'strains'.

Further difficulties arise when we attempt to name these two 'strains'. If we call both populations *T. flocculosa* var. *asterionelloides* we are subscribing

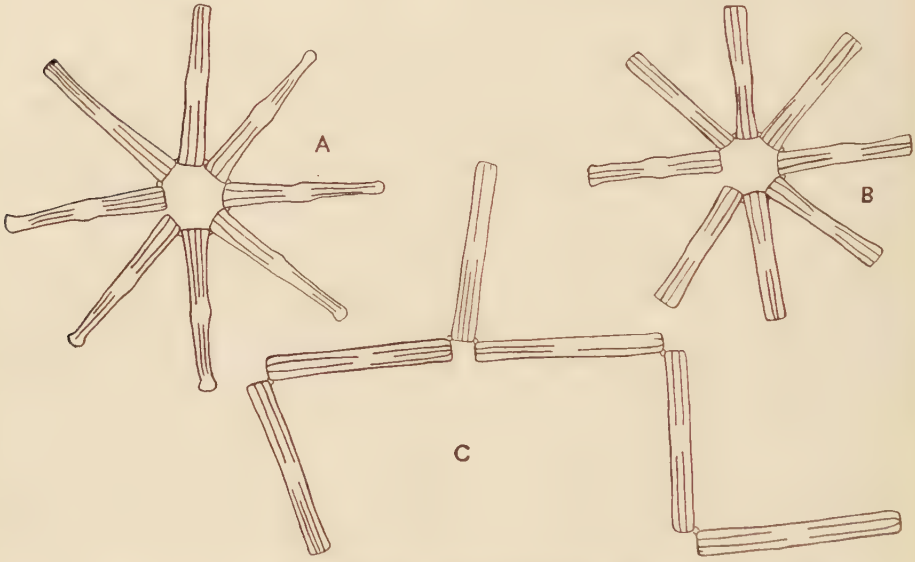

FIG. 4. A–C. *Tabellaria flocculosa*: plankton colonies from Ullswater. (×410.)

to the view that twist is unimportant as a taxonomic character. On this view it is illogical to limit the application of the name *T. flocculosa* var. *pelagica*, as Teiling (1942, p. 68) does, to colonies composed of twisted cells. If we call only the star-shaped colonies of untwisted cells *T. flocculosa* var. *asterionelloides*, then either we have to create a new taxon for those with twisted cells, or we have to include them under *T. flocculosa* var. *teilingii* from which they differ in the degree of twisting, in size of the rudimentary septa, and in the number of rays per turn of the helix.

It is obvious that strains (1) and (3) are distinct on account of their different length ranges and in respect to the angles between the cells. Should strain (3) be regarded as *T. flocculosa* var. *asterionelloides*? It should be recalled that the neotype of *T. flocculosa* var. *asterionelloides* contains similar colonies to that illustrated in Fig. 4, C, but the cells are untwisted. These difficulties will be discussed in a later section.

Another sample difficult to interpret is one from Törnetrask (1/7/1949). In addition to the characteristic parachute-shaped colonies of *T. flocculosa* var. *geniculata* (Fig. 2, E, F), corkscrew- and star-shaped colonies of non-geniculate

cells are present. Some of them (Fig. 5, D–G), by virtue of their arcuate outline and asymmetrical central swelling, show affinities to the geniculate frustules of greater valve-length (Fig. 5, A–C) and one is tempted to believe that Fig. 5, A–G, represents a morphological series of *T. flocculosa* var. *geniculata*.

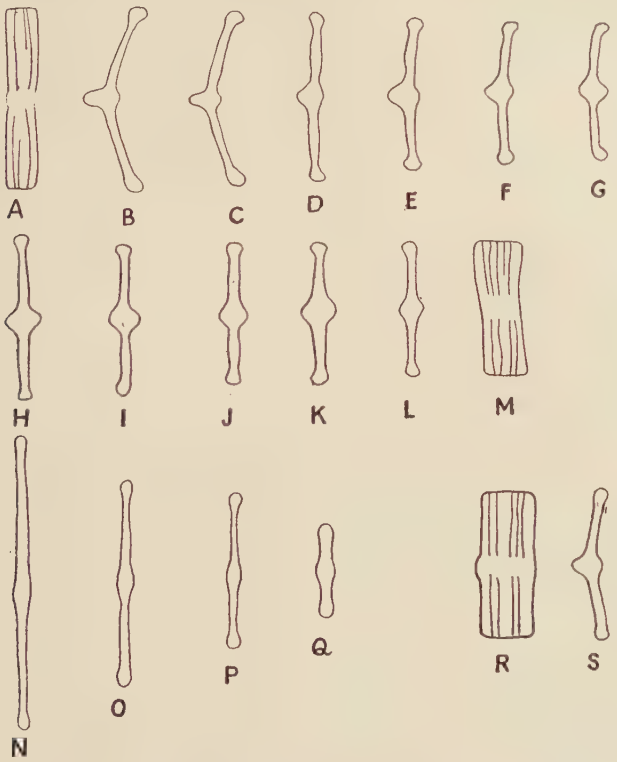

FIG. 5. A–Q, Frustules in valve and girdle view of *Tabellaria flocculosa* from the plankton of Törnetrask (for explanation see text, p. 140). R, S, *T. flocculosa* from Chicago, Illinois (drawn from H. L. Smith, spec. typ. No. 588; B.M. 26149). (×410.)

The identification of such specimens as those of Fig. 5, H–M, is more difficult. Some frustules are twisted and this may be accompanied by valve asymmetry (Fig. 5, L), but despite having twisted frustules and corkscrew colonies such specimens differ from *T. flocculosa* var. *teilingii* in the absence of rudimentary septa and in the number of rays per turn of the helix. There are also frustules in star-shaped colonies with a symmetrical valve contour but very prominent central swelling. Fig. 5 shows how difficult it is to identify specimens of valve-length 40–51 μ. No finality can be reached without recourse to other samples and/or clone cultures of *T. flocculosa* var. *geniculata* grown to low valve-lengths. Yet another population is represented in this sample (Fig. 5, N–Q), and its characters, together with those of the other postulated populations, are summarized in Table III.

TABLE III

Colony morphology.	Cell morphology.	Length range.	Figure.
(1) Parachute- or corkscrew-shaped; zigzag connexions very rare.	Geniculate or slightly arcuate. Central swelling of valve prominent and asymmetrical.	40–63 μ*	5, A–G
(2) Corkscrew-shaped or star-shaped; zigzag connexions very rare.	Slightly twisted. Central swelling of valve prominent and symmetrical.	41–52 μ	5, H–M
(3) Zigzag.	Untwisted. Central swelling inconspicuous and symmetrical.	24–89 μ	5, N–Q

* The sum of the measurements from the tip of each apical inflation to the centre of the central inflation.

The presence of two or more length groups in a diatom sample need not lead to the conclusion that two genetically distinct populations are present. We have seen (p. 134) that Huitfeldt-Kaas described a new variety (*T. fenestrata* var. *willei*) merely because in a sample containing var. *asterionelloides* 'intermediate forms could not be observed, which therefore indicates that they (*T. fenestrata* vars. *willei* and *asterionelloides*) were not merely different stages in development of the same form'. This test, however, is insufficient for the delimitation of diatom varieties because of the peculiar method of diatom multiplication. This results in a decrease in the mean size of the population, a process which is ultimately offset by the formation of auxospores. Hence the 'var. *asterionelloides*' of Huitfeldt-Kaas' sample was most likely a long-celled population derived, after a period of auxospore formation, from the 'var. *willei*'.

Variation in plankton Tabellaria from lake to lake

In the following account the colony and cell morphology of some naturally occurring plankton *Tabellaria* is described. Each description and set of drawings refers to what is believed to be (according to the morphological criteria enumerated in the previous section) a genetically homogeneous population.

1. THIRLMERE (Fig. 6, A). The colonies are either zigzag or complex stars, with or without transitions to the zigzag condition. Each 'star' consists of a flattened helix and each turn of the helix is composed of about four to six rays (each ray consists of one cell or two daughter-cells still clinging by their valve faces). Occasionally these stars contain four turns, and in this condition they come to lie parallel to the axis of the helix; they then have the appearance of a corkscrew. In such colonies there is no tendency for cells with an uneven number of septa to have more at the inner than at the outer pole. This is in contrast to the neotype of *T. flocculosa* var. *asterionelloides*.

2. CONISTON WATER (Fig. 6, B, C). The colonies show all transitions from a zigzag to a simple 'star' condition. Of particular interest are the colonies of short cells (*c.* 30 μ) which closely resemble *T. flocculosa* var. *pelagica*. They differ from *T. flocculosa* var. *teilingii* in that the cells are untwisted and the helix, at its greatest development, is only composed of two turns. If the

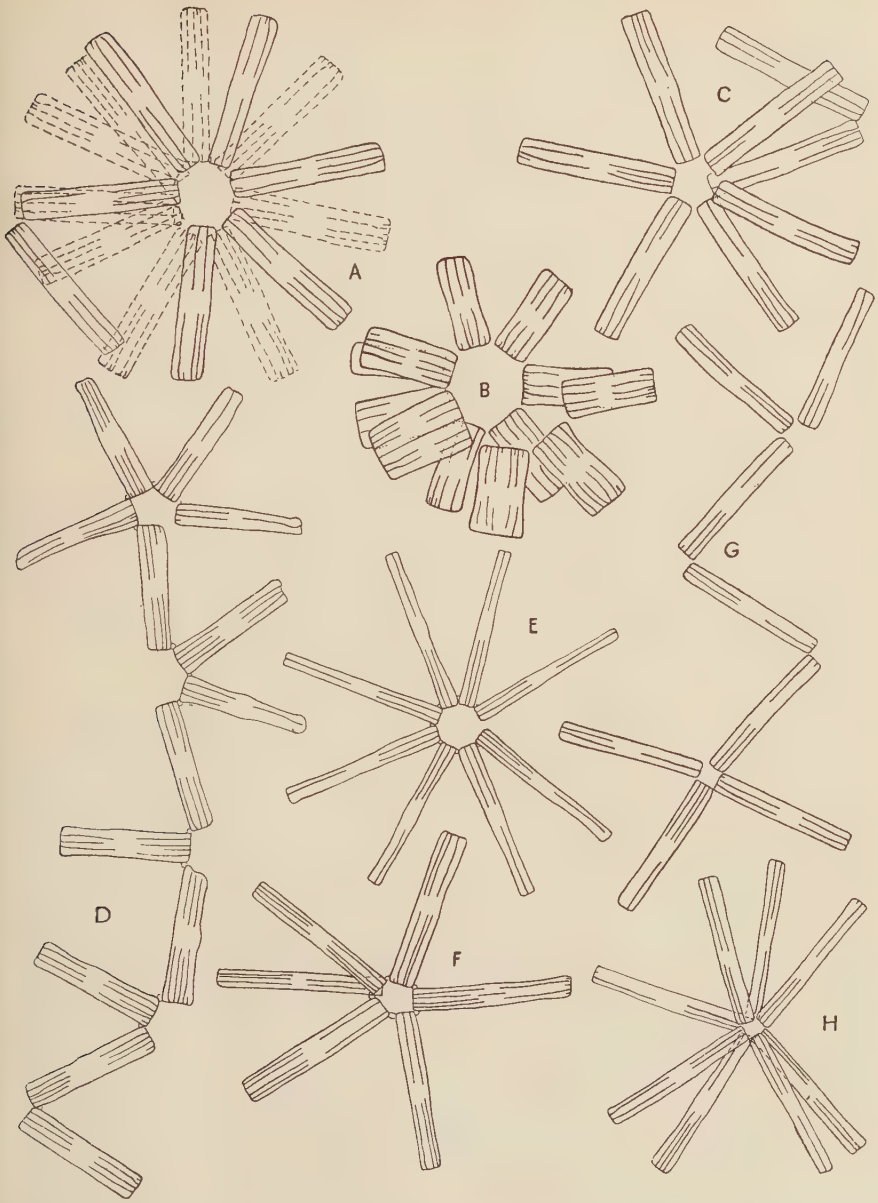

FIG. 6. A–H. Plankton colonies of *Tabellaria flocculosa* from some lakes of the English Lake District. A, Thirlmere (1951); B, C, Coniston Water (1950); D, Loweswater (1949); E, Windermere (1951); F, Windermere (1907); G, H, Windermere (1921). (×400.)

neotype of *T. flocculosa* var. *asterionelloides* is compared with the Coniston material of similar valve-length it will be seen that the Coniston samples have (*a*) a higher mean number of septa per cell, (*b*) a lower ratio of apical to pervalvar axis at any given valve-length, (*c*) no tendency to taper.

3. Loweswater (Fig. 6, d). The colonies are predominantly zigzag and the star-shaped colonies have 4–5 rays to every turn of the helix. The cells are strongly twisted and, in consequence, zigzag colonies do not lie in one plane. In star-shaped colonies the twist causes the outer ends of the cells to appear somewhat rounded in outline, owing to part of the valve face being seen.

This sample differs from the neotype of *T. flocculosa* var. *asterionelloides* in having cells with a well-marked twist.

4. Windermere (Fig. 6, e). The colonies are very uniform in morphology; 95 per cent. are star-shaped as in Fig. 6, e and the remainder are obvious derivatives.

This sample differs from the neotype of *T. flocculosa* var. *asterionelloides* in having (*a*) cells with a slight twist, (*b*) 7, 8, or 9 rays per turn of the helix.

5. Ullswater (Fig. 4, a). This differs from the neotype of *T. flocculosa* var. *asterionelloides* in having (*a*) cells with a very strong twist, (*b*) 7, 8, or 9 rays per turn of the helix.

This population is of great interest because a few colonies have as many as four turns to the helix and when seen lying with their long axis parallel to the field of the microscope they resemble *T. flocculosa* var. *teilingii*. If colonies of the same valve-length are compared, however, it will be found that they differ in the number of rays per turn of the helix and in the degree of twisting.

Variations in (*a*) the shape of the valves, (*b*) the degree of prominence of the rudimentary septa, and (*c*) shell thickness are also found between populations from different lakes. Since characters (*a*) and (*b*) vary with valve-length, only frustules of the same valve-length should be compared. Details of shell thickness have been omitted since I possess no objective means of measuring this character.

From the five accounts of plankton *Tabellaria* given above it will be realized that considerable difficulties are encountered when attempts are made to name these populations since none exactly fits the description of any existing variety. Either we have to describe them as new varieties or extend the varietal limits of those already described. All the lakes mentioned above are situated in different drainage basins in a relatively small area of north-west England (the 'Lake District') and samples from other regions have yielded plankton *Tabellaria* with yet further variations in colony and cell morphology. It is therefore very necessary to examine the various characters which have been used to diagnose plankton varieties and to see whether existing taxa do, in fact, correspond to morphologically discontinuous groups.

Seasonal variation

We have already seen (p. 132) that the neotype of *T. flocculosa* var. *asterionelloides* consists of a mixture of about 70 per cent. star-shaped colonies and 30 per cent. zigzags. It is reasonable to look for seasonal changes in the proportions of these two types of colony and, in fact, many instances of the phenomenon have been reported. Thus Schröter (1896) for Zürichsee, Wesenberg-Lund (1908) for Furesø, and Quartier (1948) for the lakes Moral,

Neuchâtel, and Bienne have described a 'seasonal dimorphism' in *T. flocculosa* var. *asterionelloides*. They observed that the chain (i.e. zigzag) form predominated in the winter months and the star-shaped form in summer. In Lough Neagh, however, Dakin and Latarche (1913) found that the star-form predominated all the year round, zigzag colonies being exceedingly rare. West and West (1909, p. 261) likewise failed to find any 'seasonal dimorphism' as a result of their monthly sampling of Windermere from 1907 to 1908.

Observations on colony morphology of plankton *Tabellaria* in five lakes are summarized in Table IV. Both net and water samples were collected regularly throughout the year, at approximate intervals of 1 week from Windermere, 2 weeks from Loweswater, and 4 weeks from Coniston Water and Ullswater. A negative record for any form means that the organism was seen neither in 100 ml. of sedimented water nor in a drop of the concentrated net sample. In none of these lakes is there a 'seasonal dimorphism', i.e. there is no regular alternation of star-shaped and zigzag colonies at different seasons of the year. Moreover, no seasonal fluctuation in the number of rays per turn of the helix has been observed.

TABLE IV

Lake.	Total No. of collections from Jan. 1949 to Jan. 1952.	Number of occasions on which *Tabellaria* was present.	Number of occasions on which star-shaped colonies were present.	Number of occasions on which star-shaped colonies predominated over zigzag colonies of same population.
Thirlmere . .	40	38	26	1
Coniston Water .	40	40	17	0
Loweswater . .	79	76	76	20
Windermere . .	158	145	145	122
Ullswater* . .	49	49	49	47

* These results refer only to populations 1 and 2 in Table II.

Variations in the mean number of septa and in the apical/pervalvar axis ratio occur in cells of the same strain and length group at different times, though no seasonal regularity in this phenomenon has been observed. In June 1948 a sample of plankton *Tabellaria* from Esthwaite showed an apical/pervalvar axis ratio of $9{\cdot}3\pm2{\cdot}4$ and $4{\cdot}2\pm1{\cdot}4$ septa per cell. In May 1949 cells of the same strain and length showed an apical/pervalvar axis ratio of $8{\cdot}7\pm2{\cdot}8$ and $3{\cdot}6\pm0{\cdot}9$ septa per cell, each value being the mean and standard deviation of fifty determinations. The differences in the mean number of septa have been found to be due to variations in the number of septa in the dividing cell. Evidence of this is obtained by making observations on (*a*) the maximum number of septa per cell, and (*b*) the minimum number of septa in rays consisting of two daughter-cells still adhering by their valve faces.

Let us suppose that single cells have a maximum of four septa and that the minimum total number of septa in a pair of adjacent daughter-cells is four.

Provided a sufficient number of cells is examined, this would mean that cell division is always taking place when the mother-cell contains four septa. Suppose now that single cells contain up to six septa, and that the minimum total number of septa in a pair of adjacent daughter-cells is still four. This suggests that some cells are dividing with four septa (because a pair of adjacent daughter-cells has a total of four septa), whereas others are dividing with more than four septa (because single cells contain up to six septa).

This phenomenon can be expressed quantitatively by calculating the following expression. In a given sample let x represent the number of pairs of daughter-cells containing a total of four septa and y the number of single cells containing more than four septa. Then the 'septa index' is $100x/(x+y)$ per cent. If the whole population is dividing when the mother-cell contains four septa, the 'septa index' is 100 per cent. If all the cells are dividing with more than four septa the 'septa index' is 0 per cent.

Comparisons of 'septa indices' should, of course, only be made between populations of the same (and preferably, narrow) length range and of approximately the same length frequency. This is because there is a tendency in *T. flocculosa* for the mean number of septa to increase with decrease in valve length (see Table V). In Esthwaite Water in June 1948 the 'septa index' was 14 per cent. and in May 1949, 80 per cent. These results relate to the same strain and are estimated for cells of the same length range (44–57 μ).

Long-term variation in plankton Tabellaria

Thanks to the foresight of those responsible for founding the Freshwater Biological Association, phytoplankton samples have been collected from Windermere at monthly intervals since 1934 and at fortnightly intervals since 1938, and this forms the most complete series from a single lake available to me. In addition, Windermere phytoplankton samples collected by W. and G. S. West (Sept. 1907–Aug. 1908), W. H. Pearsall (April 1921; Sept. and Nov. 1922; Aug. 1928; Sept. 1929; Sept. 1931 and Sept. 1932), and P. M. Jenkin (March, April, May, and June 1932) are extant. If we compare these early samples with those obtained today, striking differences are apparent. Thus, in the samples collected by the Wests in 1907 and 1908 (Fig. 6, F) the cells show no well-defined twist, and in the stellate colonies there are five or six (very rarely seven) rays in a complete turn of the helix. Such colonies are illustrated in West and West (1909, pl. VII). In the samples collected by Pearsall in 1921 the cells are again untwisted, but in the majority of the stellate colonies there are four rays in a complete turn of the helix, rarely five or six (Fig. 6, H). Strongly twisted cells (comparable with those from Ullswater illustrated in Fig. 4, A) are found in the samples taken in April 1932. From then until the time of writing there do not appear to have been any significant changes in cell or colony morphology. It is therefore obvious that such characters as twisting and number of rays to a turn of the helix are not stable in the plankton *Tabellaria* of Windermere. This militates against their being used for diagnostic purposes.

IV. The Taxonomic Significance of Various Characters in Delimiting Plankton Varieties of *Tabellaria*

1. *Colony morphology*. At first sight the star-shaped colonies of *T. flocculosa* var. *asterionelloides* appear to be very different from the corkscrew type of *T. flocculosa* var. *teilingii*, but when the two are more closely examined it will be found that they are not fundamentally distinct. Both are really like screw-threads, differing in number of turns of the helix and in coarseness of pitch. Thus in *T. flocculosa* var. *asterionelloides* the number of turns is usually small and the pitch fine, whereas in *T. flocculosa* var. *teilingii* the number of turns is usually large and the pitch coarse.

The description of a plankton population of *Tabellaria* as star- or corkscrew-like depends chiefly on the degree of fragmentation of the colonies and the coarseness of the pitch. This is because colonies of fine pitch and/or small number of turns come to rest on the girdle surfaces of the lowermost turn of cells, thus giving the appearance of a star, whereas colonies of coarse pitch and/or large number of turns come to rest on the tips of their cells and give the impression of a corkscrew. Thus Huitfeldt-Kaas (1906), whose drawing (Fig. 2, c) of *T. flocculosa* var. *pelagica* illustrates a star-shaped colony, states, nevertheless, in the text that 'the star-shaped colonies, when fully developed, form several, sometimes four to five, spirally-wound coils, on account of which the colony seen from the side reminds one very much of a screw-thread'.

In the plankton *Tabellaria* of Windermere (see p. 144) colonies with three or four turns of the helix are found, but they never have a corkscrew appearance because the pitch is fine and the colonies always come to rest on one end and hence appear star-shaped. In Thirlmere (see p. 142) colonies with a similar number of turns to the helix have a coarser pitch and therefore they sometimes come to rest on their side and appear to be corkscrew-like.

In Ullswater, strain (1) (Table II) usually forms star-shaped colonies composed of one and a small fraction of a turn, but occasionally colonies of four turns are formed, and these, having a coarse pitch, come to rest in such a position that they resemble a corkscrew. We may conclude, therefore, that these two types of colony morphology have no taxonomic significance.

It is of interest to note that no plankton strain of *Tabellaria* has yet been found in which the colony morphology is invariably of helical form. All samples which I have examined (including *T. flocculosa* var. *teilingii* and *T. flocculosa* var. *geniculata*) show some tendency to a reversal of polarity in mucilage-pad formation between daughter-cells, a phenomenon which results in occasional or frequent transitions to zigzag form.

2. *Frustule symmetry*. We have already seen (p. 136) that Teiling regards frustule twist as diagnostic of *T. flocculosa* var. *teilingii* (his *T. flocculosa* var. *pelagica*) but that the character is also found in plankton populations (Ullswater (1) and (3), Table II), which would not be referred to that taxon.

Moreover, observations on Windermere and Ullswater have shown that the dominant plankton form of *Tabellaria* has, at various times, consisted of cells with (*a*) no twist, (*b*) a slight twist, and (*c*) a strong twist. For this reason I do not regard frustule twist as a good taxonomic character in diagnosing varieties of plankton *Tabellaria* in the English Lake District. This is not to deny that in other lakes (e.g. in those for which *T. flocculosa* var. *teilingii* has been recorded) frustule twist may be such a genetically stable factor that it has characterized plankton populations of *Tabellaria* for thousands of years.

Polarity of septa formation has been mentioned on p. 142. In helical colonies such a polarity may manifest itself in a tendency for there to be more septa at the inner than at the outer end of the cell. In a sample of plankton *Tabellaria* from Windermere in September 1951 (a sample in which 99 per cent. of the colonies were star-shaped) 81 per cent. of the cells with an uneven number of septa *in star-shaped colonies* had more at the inner than at the outer end of the cell. This fact suggests that the first septum to be developed after cell-division forms at the inner pole of the cell, i.e. at the end where the mucilage pad is produced.

3. *The apical/pervalvar axis ratio and the number of septa per cell.* The importance of these as taxonomic characters has been tacitly acknowledged by all those authors who have ascribed new plankton varieties to *T. fenestrata* or to *T. flocculosa*. The widespread belief, now shown to be erroneous (Knudson, 1952), that *T. fenestrata* is diagnosed by its higher apical/pervalvar axis ratio and smaller number of septa accounts for the original attribution of var. *pelagica* to the species *T. flocculosa* and the other plankton varieties to *T. fenestrata*.

Since it has now been shown that all these taxa belong to *T. flocculosa*, the existence of varieties characterized by distinctive apical/pervalvar axis ratios and/or range of septa numbers must now be queried. To demonstrate such differences it is essential to compare populations over the same, and, if possible, a large part of their length range. Unfortunately it is not possible to reach a conclusion merely by recourse to the original authors' type material, drawings, or descriptions; this is either because information is so sparse or because the original material covered only a very small part of the length range of the variety. The validity of these characters must therefore be tested on other populations which are more completely known. Table V is based on results obtained from various Lake District populations and also from *T. flocculosa* var. *teilingii* (from Täftlången). Each value is the mean of twenty determinations, those marked * being derived from unialgal clone cultures maintained for approximately 2 years in the laboratory (Knudson, 1952). The apical/pervalvar axis ratios do not alter significantly in culture, and the fact that different strains retain their characteristic apical/pervalvar axis ratios when grown under identical cultural conditions suggests that these ratios are genetically determined, though subject to a small amount of environmental modification (cf. p. 145). In compiling Table V great care was taken to ensure that each set of values refers to only one strain.

Table V

	Apical/pervalvar axis ratios					Number of septa per cell				
	12·7–15·9 μ	19·0–22·2 μ	28·5–31·7 μ	44·4–47·6 μ	60·2–63·4 μ	12·7–15·9 μ	19·0–22·2 μ	28·5–31·7 μ	44·4–47·6 μ	60·2–63·4 μ
1. Thirlmere (p. 142)	—	—	2·1±0·3	5·6±0·9	6·7±1·1	—	—	8·3±1·2	4·4±1·0	5·3±0·9
	—	—	—	5·7±1·8*	—	—	—	—	4·2±1·2*	—
2. Coniston Water (p. 142)	—	—	2·3±0·5	5·0±0·9	8·0±2·1	—	—	8·4±1·7	5·0±1·2	4·6±1·3
	1·2±0·4*	1·6±0·4*	2·8±0·9*	5·6±1·5*	—	9·0±2·2*	8·3±1·5*	7·0±2·1*	5·5±0·8*	—
3. Loweswater (p. 144)	—	—	—	7·6±1·6	—	—	—	—	4·3±1·3	—
	1·6±0·3*	1·9±0·5*	4·7±0·9*	7·8±2·2*	—	9·0±2·0*	9·4±1·6*	5·5±1·1*	4·7±1·2*	—
4. Windermere (p. 144)	—	—	—	9·7±0·8	12·1±2·3	—	—	—	3·9±0·5	3·6±0·5
	—	—	3·8±1·6*	10·3±2·9*	—	—	—	5·2±1·2*	3·4±1·0*	—
5. Ullswater (strain 1 in Table II)	—	—	—	8·7±0·9	—	—	—	—	2·8±1·2	—
	—	—	—	9·9±2·3*	—	—	—	—	3·6±0·8*	—
6. Ullswater (strain 2 in Table II)	—	—	—	8·4±1·9	—	—	—	—	3·4±1·1	—
	1·8±0·4*	3·8±0·8*	8·2±1·8*	—	—	7·5±1·4*	5·6±1·1*	4·7±0·9*	—	—
7. Täftlången (p. 137)	—	1·9±0·3	2·9±0·5	—	—	—	6·6±1·3	5·8±1·6	—	—
	—	—	—	—	—	—	—	—	—	—
8. Hästefjord (p. 132)	—	—	—	—	12·7±1·2	—	—	—	—	?
	—	—	—	—	—	—	—	—	—	—

It is obvious from Table V that the different strains vary more in apical/pervalvar axis ratio than in mean number of septa. The ratios are represented in Fig. 7, from whence it can be seen that the 'size curve' may be characteristic of a given strain. Although the differences between certain of the size curves are highly significant, it is obvious that this property cannot be used except in

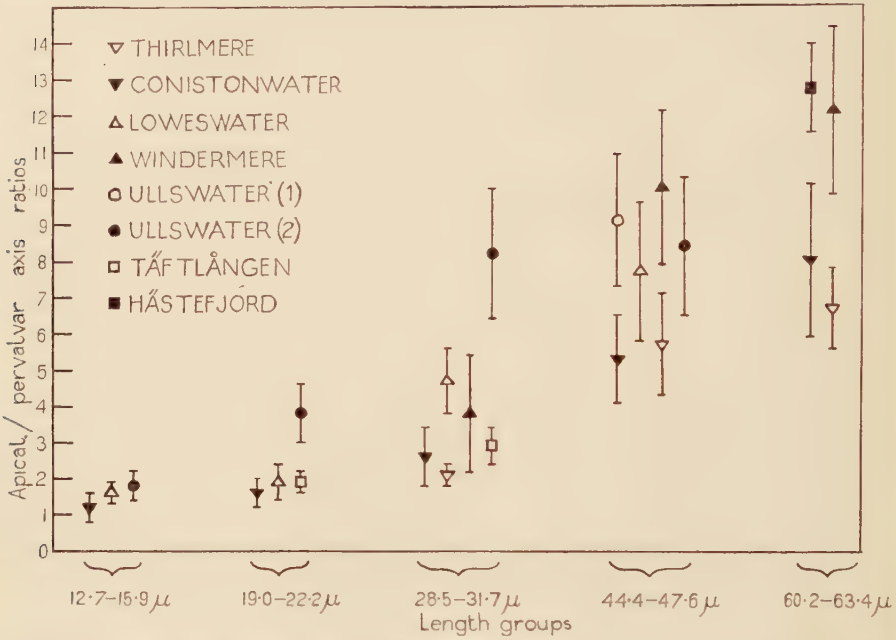

Fig. 7. Relationship between the apical/pervalvar axis ratio and valve-length in various plankton strains of *Tabellaria flocculosa*. Each value is the mean of twenty determinations, except where results from natural and cultural populations are available (see Table V), in which case the mean is based on forty determinations. The standard deviations of the means are indicated.

a qualitative manner, as a taxonomic character. There seems little doubt, however, that strains of *T. flocculosa* may differ ontogenetically in apical/pervalvar axis ratio.

Due consideration must be given to these phenomena in attempting to delimit varieties of *T. flocculosa*. Thus the fact that Meister's drawing of *T. fenestrata* var. *lacustris* shows cells with six septa does not, *ipso facto*, make it distinct from the neotype of *T. flocculosa* var. *asterionelloides* in which cells of the same valve-length have four (occasionally five) septa.

4. *The length range.* Since no lake with plankton *Tabellaria* has yet been found without a *Tabellaria* flora attached to its shores, and since such *Tabellaria* is detached by wave-action, it is inevitable that all plankton collections are contaminated to some extent by cells of non-plankton origin. Such cells may cause considerable trouble when attempts are made to determine the natural limits of the length range of plankton *Tabellaria*.

Measurements of valve-length were made on the Windermere plankton *Tabellaria* from 1934 onwards. Two samples per year were selected (usually May and October) at times when sufficient *Tabellaria* was present for the valve-lengths of fifty colonies to be counted with ease. Any colonies with atypical cell or colony morphology were excluded, i.e. the measurements refer to populations which were obviously different from the shore form. During the period 1934–51 the maximum valve-length of the Windermere plankton *Tabellaria* was 79 μ. The minimum valve-length of colonies which could with certainty be identified as part of the same series was 38 μ. This poses the question, Does 38 μ represent the valve-length at which cells of the Windermere kind of plankton *Tabellaria* lose their viability? Or does it merely represent the point at which it ceases to be distinguishable from *T. flocculosa* var. *flocculosa*? This problem has been investigated by means of uni-algal clone cultures. A colony of valve-length 51 μ isolated from Windermere in December 1948 has yielded a clone which at the time of writing (December 1951) measures 30 μ. Plankton strains from other lakes have already reached even smaller dimensions. Thus the clone culture derived from a colony of valve-length 40 μ, resembling Fig. 6, D, isolated from Loweswater in May 1949 now measures 14 μ; a colony of valve-length 44 μ resembling Fig. 4, B, isolated from Ullswater in October 1949 now exists at a valve-length of 6 μ, and a clone culture from Coniston Water, which measured 51 μ when isolated in January 1950, has produced cells of valve-length 8 μ.

These results with clone cultures show that populations such as Ullswater (2) in Table II are viable at valve-lengths as low as, or lower than, those of *T. flocculosa* var. *teilingii*. The most remarkable morphological characteristic of Teiling's material is that the peculiar colony morphology is maintained to such low valve-lengths. In this respect it appears to differ from all other plankton *Tabellaria* which I have so far examined. The minimum valve-lengths of *c.* 33 μ often quoted for *T. flocculosa* var. *asterionelloides* are therefore artificial ones due to the fact that below this valve-length the colonies cannot with certainty be distinguished from *T. flocculosa* var. *flocculosa*.

V. Discussion and Conclusions

Owing to the extreme variability of *T. flocculosa*, morphologically distinct populations are not difficult to find, and the early part of this paper gives examples of some plankton populations. The characters by which they may differ are summarized below:

1. *Colony morphology*
   - (*a*) helical or zigzag; tendency for either type to predominate;
   - (*b*) angle between the cells (which determines the number of rays to a complete turn of a helical colony);
   - (*c*) coarseness of pitch (in helical colonies);
   - (*d*) number of cells to the colony;
   - (*e*) number of turns to the helix (this is, of course, the resultant of $b+d$).

2. *Cell morphology*
   (*a*) twisted or not;
   (*b*) geniculate or not;
   (*c*) tapering or not;
   (*d*) presence or absence of polarity of septa formation in relation to mucilage pad formation between daughter-cells;
   (*e*) thick or thin shell;
   (*f*) apical/pervalvar axis ratio;
   (*g*) maximum and minimum number of septa per mature cell;
   (*h*) degree of prominence of rudimentary septa;
   (*i*) length range;
   (*j*) valves: degree of prominence of central and terminal inflations.

With the possible exception of 2 (*b*) these characters appear in different combinations in different lakes and this makes a natural classification of the plankton *Tabellaria* a matter of great difficulty. The results of culture work show that the majority of these variations are genetically controlled, not conditioned by the environment. In interpreting their taxonomic significance we have to consider the peculiar life-history of the diatoms. Their normal method of reproduction is by binary fission, and in most species, including those of *Tabellaria*, this results in a gradual diminution of valve size. At considerable intervals another type of reproduction (auxospore formation) occurs in which the maximum valve size is restored. In some diatoms auxospores are produced by syngamy (fusion of two gametes to form a zygote), but in many there is autogamy (fusion of two nuclei derived from the same parent cell). It is not known which method occurs in *Tabellaria*.

In plankton *Tabellaria* there are normally very great fluctuations in numbers of the population between one auxospore formation and the next. This means that the number of clones (using that term to represent the products by binary fission of a single auxospore) represented in the population when it is numerous is very few compared with the number of individuals, perhaps so few that they correspond to the different recognizable strains, although this is not very probable. On the other hand, the clones making up a population are different from those making up the population, say, 5 years previously. If the process of auxospore formation in *Tabellaria* involves any reassortment of genes, the alterations with time in the morphology of the plankton *Tabellaria* in a single lake become explicable.

Another relevant consideration on which we have no information is the extent to which the population of a single lake or drainage basin is self-contained. It seems unlikely that the interchange of genes between one drainage basin and another is more than very occasional, although the widespread distribution of most diatom species might be regarded as evidence to the contrary.

Although the last three paragraphs have done little more than reveal the depths of our ignorance, they do set out the reasons for taking a wide view of

the concept of the variety in diatoms in general and plankton *Tabellaria* in particular. It is now generally accepted that fluctuating variations (i.e. those due to fluctuations in valve-length) should not be given varietal rank and insistence on genetic distinctness is now increasing (Hustedt, 1937, p. 192). But genetic distinctness can only be inferred from a comparatively regular association of a number of characters in a geographical area or a particular type of environment over a considerable period.

Further work needs to be done on *T. flocculosa* vars. *geniculata* and *teilingii*. The difficulty of identifying small specimens of *T. flocculosa* var. *geniculata* has been mentioned on p. 140, and it will be interesting to know whether any variations in cell or colony morphology can be detected in specimens of *T. flocculosa* var. *teilingii* from different lakes. The discovery in Ullswater and Törnetrask of colonies resembling *T. flocculosa* var. *teilingii* in colony morphology and frustule twist but not in other characters is noteworthy. Slightly geniculate frustules of *T. flocculosa* are present in a sample from Chicago, Illinois (H. L. Smith, spec. typ. No. 588; B.M. 26149), but these may have been merely ephemeral monstrosities (Fig. 5, R, S) and their arrangement in zigzag colonies does not necessarily invalidate any evidence for the morphological discontinuity of *T. flocculosa* var. *geniculata*.

It is interesting to note that geographical and ecological evidence strengthens the claims of these two taxa to varietal status; Teiling (1944, p. 42, and 1947, p. 230) states that both these taxa are endemic to Scandinavia and that *T. flocculosa* var. *teilingii* is confined to oligotrophic lakes. The traditional idea of a diatom variety as a taxonomic group distinguishable by morphological means alone may have to be replaced by a broader one in which physiological, ecological, and geographical data are included. Only in this way can a natural classification be achieved.

The earlier parts of this paper show the difficulty of defining precise morphological limits to *T. flocculosa* var. *asterionelloides*. If all stellate colonies and no others are included, a very artificial group is made and there are no grounds for retaining either *T. fenestrata* var. *lacustris* or *T. fenestrata* var. *willei*.

The following summarizes my conclusions on the morphological delimitation of plankton varieties in *T. flocculosa*:

1. *T. flocculosa* var. *pelagica* Holmboe is not necessarily synonymous with *T. flocculosa* var. *pelagica* Holmboe emend. Teiling, and only the discovery of Holmboe's type material can settle this without any doubt. In the meanwhile it seems desirable to distinguish between the two, and I have consequently given Teiling's material the name *T. flocculosa* var. *teilingii*.

2. The greatest degree of morphological discontinuity is shown by (*a*) *T. flocculosa* var. *geniculata* and (*b*) *T. flocculosa* var. *teilingii*, but it has not yet been demonstrated that these are genetically stable groups, and some of the characters on which these taxa are diagnosed have been found differently combined in other populations.

3. *T. flocculosa* var. *asterionelloides*, *T. fenestrata* var. *lacustris*, and *T. fenestrata* var. *willei* are synonymous. The question of whether *T. flocculosa* var.

asterionelloides and *T. flocculosa* var. *pelagica* are synonymous with *T. flocculosa* var. *flocculosa* is postponed for consideration in a later paper, but it is clearly unnatural to separate into varieties plankton populations of *T. flocculosa* on the basis of whether their colonies are star-shaped or zigzag.

Acknowledgements

My thanks are due to the Director and Staff of the Freshwater Biological Association and particularly to Mr. G. J. Thompson, under whose very able supervision phytoplankton samples have been collected since 1934. I am grateful to Dr. E. Teiling for material from Täftlången and Dr. C. H. Mortimer for collections from Törnetrask. I should also like to thank Professor W. H. Pearsall, F.R.S., for valuable suggestions, Mr. and Mrs. R. Ross for the Latin diagnosis, and Mr. R. Ross for his expert advice and generous assistance in the preparation of this paper.

Summary

1. The five known plankton varieties of *Tabellaria* have been shown to belong to the species *T. flocculosa.*

2. Specimens of plankton *Tabellaria* from all the lakes of the English Lake District have been examined. They do not agree with descriptions of existing varieties.

3. Long-term observations on natural populations and clone cultures show that morphological differences are the result partly of environmental and partly of genetical factors.

4. The summated morphological difference between populations is due to different combinations of at least fifteen variable characters. The number of known combinations of these characters increases as more and more plankton samples are investigated.

5. Morphologically distinct populations of plankton *Tabellaria* are found in (*a*) a single sample, (*b*) the same lake at various times, and (*c*) different lakes.

6. It is not advisable to grant each morphologically distinct population varietal status even when the differences are shown to have a genetic basis. A recommendation is made that plankton populations of *T. flocculosa* should only be granted varietal status when it has been shown that they are morphologically (and therefore genetically) stable over long periods of time.

7. On this basis, *T. flocculosa* var. *geniculata* and *T. flocculosa* var. *pelagica* Holmboe emend. Teiling (now renamed *T. flocculosa* var. *teilingii*) have the strongest claims to retain varietal rank.

8. There are no morphological grounds for regarding *T. flocculosa* var. *asterionelloides*, *T. fenestrata* var. *willei*, and *T. fenestrata* var. *lacustris* as distinct plankton varieties.

Literature Cited

Dakin, W. J., and Latarche, M., 1913: The Plankton of Lough Neagh. Proc. R. Irish Acad. B, xxx, No. 3, pp. 20–96.

Huber-Pestalozzi, G., 1942: Das Phytoplankton des Süßwassers. Die Binnengewässer, Teil ii, 2. Hälfte, Stuttgart.

Huitfeldt-Kaas, H., 1906: Planktonundersøgelser i norske vande. Christiania.

Hustedt, F., 1931: Bacillariophyta. Rabenhorst, Kryptogamen-Fl. vii, Teil 2.

—— 1937: Zur Systematik der Diatoméen I. Ber. dtsch. bot. Ges. lv (3).

Knudson, Brenda M., 1952: The Diatom Genus Tabellaria. I. Taxonomy and Morphology. Ann. Bot., n.s., xvi, No. 63, pp. 421–40.

Quartier, A., 1948: Sur le comportement de Tabellaria fenestrata (Lyngb.) Ktz. dans les trois lacs sub-jurassiens. Schweiz. Z. Hydrologie, x (4), 13–22.

Schröter, C., 1896: Die Schwebeflora unserer Seen (Das Phytoplankton). Neujahrsbl. naturf. Ges. Zürich, xcix.

Teiling, E., 1942: Schwedische Planktonalgen 3. Bot. Notiser, 1942, pp. 63–68.

—— 1944: Våra viktigare fytoplankter. Medlemsbl. för biologilärarnas Förening.

—— 1947: Staurastrum planctonicum and St. pingue. A Study in Planktic Evolution. Svensk. bot. Tidskr., Bd. xli, H. 2 pp. 218–34.

Thunmark, S., 1945: Die Abwasserfrage der Växjöseen in hydrobiologischer Beleuchtung. Meddelanden från Lunds Universitets Limnologiska Institution. Lund.

Wesenberg-Lund, C., 1908: Plankton Investigations of the Danish Lakes. General Part. Copenhagen.

West, W. and G. S., 1905: A Further Contribution to the Freshwater Plankton of the Scottish Lochs. Trans. Roy. Soc. Edin., xli, pt. iii (No. 21).

—— 1909: The Phytoplankton of the English Lake District. Naturalist, Lond., dcxxvi. 115–22; dcxxx. 260–7.

A Physiological Study of Embryo Development in *Heracleum sphondylium* L.

III. The Effect of Temperature on Metabolism[1]

BY

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With seven Figures in the Text

Abstract

Low temperature brings about the conversion of storage proteins into soluble nitrogenous compounds in the seed of *Heracleum sphondylium*. The possible mechanism of this action and its importance in relation to the phenomenon of after-ripening are discussed.

Introduction

THE moist seeds of *Heracleum sphondylium* will only germinate after a period of 2 to 3 months at low temperature. During this time the embryo develops from a minute ball of cells weighing 0·4 per cent. of the seed dry weight to a germling weighing 30 per cent. seed dry weight. Prior to germination, therefore, a large amount of food material is transferred from the endosperm to the embryo, and previous work on this species has shown that this transfer is controlled by temperature (Stokes, 1952 *a* and *b*).

In this paper the changes in the constituents of the endosperm and embryo are compared at low temperature and room temperature.

Procedure

Both quantitative and qualitative methods were used to determine changes taking place in insoluble and soluble nitrogen, carbohydrates, fats, and acid content. Changes in dry weight were also recorded as a check on any total loss occurring through respiration.

Overall changes were followed in the whole seed, and analyses were also made separately on endosperm and embryo, but it should be mentioned that in the early stages of development the embryo only accounts for 0·4 per cent. of the seed dry weight. The initial changes in the embryo appear to be insignificantly small in comparison and the degree of error is very high.

For the analyses 10 sets of 500 seeds were dissected from the fruits and

[1] This work was presented as part of a thesis submitted for the degree of Doctor of Philosophy at the University of Bristol, 1950.

[Annals of Botany, N.S. Vol. XVII, No. 65, January, 1953.]

laid out on damp filter-paper covered with glass plates. Of these, 4 sets were placed in a refrigerator at a temperature of from 2° to 5° C. and 5 sets were stored in a room temperature of 15° C. The remaining set was analysed immediately after dissection. One set from 2° and one set from 15° were removed for analysis after 3, 6, 9, 12 weeks and after 15 weeks at 15° C.

When a set was removed for examination, half the seeds (a known number of about 250) were dried with filter-paper to remove the surface moisture present, and weighed in the imbibed condition. They were then dried in an oven at 80° C. to constant weight. From these weighings the average wet weights, dry weights, and water content per seed were calculated.

The embryos of the remaining half set were excised under standard conditions. Embryos and endosperms were then dried separately to constant weight at 80° C. Only dry weights of endosperms and embryos are available, as during the prolonged process of excision there was a considerable loss of moisture from those already dissected, especially from the embryos.

These analyses were made in 1947 and the endosperm and whole seed material was analysed in duplicate throughout. The amount of embryo material available was, however, too small to enable duplicate determinations to be made. Analyses of endosperms and embryos were, therefore, repeated with seed of the 1948 harvest.

Germination counts were made at the time of removal of the sets for examination and checked against a control set kept at 2° C.

Method (Quantitative Analyses)

After being dried to constant weight at 80° C. the seeds and endosperms were ground up in a small coffee mill to a fine powder which was re-dried to constant weight. The dried seed material available had a dry weight of approximately 1 g.

0·5 g. were suspended in 50 ml. of distilled water, and extracted for 15 minutes in a water-bath at 80° C. The soluble matter was filtered off through asbestos in a Gooch crucible at the pump. After the removal of the filtrate, the residue was washed clean from adhering soluble matter and then dried in the oven at 80° C. to constant weight.

Similar procedure was used for analysis of the endosperms, and embryos where the material was sufficient to make an extraction of soluble matter, as in the sets at 2° C. for 12 and 15 weeks.

The various fractions were estimated as follows:

1. *Nitrogen fractions*

Soluble nitrogen was determined in whole seeds and endosperms. Duplicate aliquots of 5 ml. of the filtrate were used for micro-Kjeldahl estimation.

Insoluble nitrogen was also determined for whole seeds and endosperms. Duplicate samples of about 50 mg. of the washed and dried residue were used for micro-Kjeldahl estimation.

Total nitrogen was determined for whole seeds and embryos, but not endosperms. Duplicate samples of approximately 50 mg. of the original dry seed material were used. All the embryo material available was used for one determination, except in the cases of the sets of 2° C. for 12 and 15 weeks where duplicate determinations were possible. The embryos from these were ground and treated in the same way as seed material.

Nitrogen was estimated by the modified Kjeldahl method of Chibnall, Rees, and Williams (1943). Duplicate samples in each case were incinerated with 5 ml. of concentrated sulphuric acid for 5 hours after the solutions had 'cleared'. Long-necked 200-ml. Kjeldahl flasks were used. The ammonia liberated was then distilled off from alkaline solution in 90 ml. of steam made up to 100 ml. and diluted appropriately for Nesslerization with a reagent according to Vaneslow (1940). Colour intensity was measured 15 minutes after the addition of the Nessler's reagent to the solution in a 'Spekker' absorbtiometer using 2-cm. cells and the violet (Ilford No. 601) filter, with distilled water as a reference standard. Duplicate readings were made and were usually within less than 0·002 of a scale reading, i.e. to 0·004 mg. nitrogen.

2. *Fat content*

Fat content was determined for whole seeds and endosperms. The remaining original dry matter was used for this purpose.

Samples of dry matter in extraction thimbles were extracted to constant weight with pure ether in a Soxallette distillation apparatus. Loss of weight represents the amount of ether-soluble constituents present. In view of the small quantities involved the usual procedure of weighing the extract itself was impracticable. Great care was therefore taken to ensure that accurate estimation of loss of weight of the thimbles could be determined. Weighings were made with an accuracy of 0·1 per cent.

3. *Carbohydrate fractions*

Ten ml. of the filtrate were hydrolysed with 1 ml. of 2N sulphuric acid (i.e. 0·2N acid strength) for 15 minutes in a boiling water-bath, neutralized with 2 ml. of 1N sodium hydroxide, and made up to 25 ml. This hydrolysed filtrate was used for the determination of soluble carbohydrates as simple reducing sugars.

Reducing power was estimated by the micro-method of Somogyi (1945). *Total reducing power* was determined directly on duplicate 5-ml. aliquots of the hydrolysed filtrate with an accuracy of 0·0001 mg. of reducing sugar.

Five ml. of a 30 per cent. washed yeast suspension was then added to the remaining 15 ml. of the hydrolysed filtrate and maintained in a water-bath at 35° C. for 2 hours with frequent shaking. The yeast was then centrifuged off and the *non-fermentable reducing power* estimated on duplicate aliquots of the clear solution. In no case were any non-fermentable reducing substances found to be present.

4. *Titratable acid content*

Acid content was determined on duplicate aliquots of 10 ml. of non-hydrolysed filtrate to which 3 ml. of N/50 hydrochloric acid had been added. The solution was titrated with sodium hydroxide, a Cambridge pH meter being used to record the change of pH from 2·8 to 7·8 which is the range covered by all organic acids likely to be present. Results are expressed as ml. equivalent to N/50 hydrochloric acid per seed.

All determinations were fully controlled by the use of blanks.

Qualitative Methods

Storage carbohydrates

Forty seeds were examined weekly for the distribution of starch and dextrins from each temperature. Sections of seeds were stained in iodine and results expressed according to the following criteria:

1. No coloration—no starch or dextrins present.
2. Red-brown coloration, intensity increasing with development—dextrins present in increasing quantities.
3. Blue or purple coloration and presence of starch granules—starch present.

The disappearance of storage polysaccharides and hemicelluloses from the endosperm cell walls was also observed microscopically.

Protein

Sections of seeds were stained by the Sakaguchi reaction for arginine, according to Baker (1947). A pink-red coloration showed the distribution of arginine, and since the presence of free arginine is improbable, it may be taken to indicate the distribution of proteins.

Acid content

pH gradients within the seed were observed microscopically with the use of indicators.

Expression of Results

Germination

All results in this paper are expressed as being for the 'average seed'. In all samples the germination at 2° followed the course of a control set in which the length of low-temperature treatment necessary for the after-ripening of 50 per cent. of a set was 9 weeks. At 2° C. the emergence of the radicle takes place 3 weeks after the completion of the necessary low-temperature treatment. Seeds are therefore described as after-ripened after 9 weeks at 2° and germinated after 12 weeks at 2° C. (Stokes, 1952 *a*).

Only 2 instances of germination were found from all the 4,000 seeds stored at 15° C.

Chemical analyses

Complete tables of analytical results are given in the Appendix, and are expressed both as mg. per seed and as percentage of seed dry weight. The latter method allows for the fluctuations in dry weight incurred in sampling, and since respiration losses are not large the results are on a comparable basis and are discussed in this form.

Results

Table I

Dry Weight, Mg./Seed

Weeks.	Whole seed.	Endosperm.	Embryo.	Endosperm + embryo.
		Seed of the 1947 Harvest at 2° C.		
0	3·705	4·060	0·0159	4·076
3	3·652	3·871	0·0181	3·889
6	3·551	4·025	0·0551	4·080
9*	3·850	3·770	0·1880	3·958
12	3·534	3·370	1·4640	4·834
		Seed of the 1947 Harvest at 15° C.		
0	3·705	4·060	0·0159	4·076
3	(Set discarded because of excessive infection)			
6	3·479	3·678	0·0230	3·701
9	3·603	3·406	0·0260	3·431
12	3·821	3·665	0·0299	3·695
15		3·616	0·0396	3·656
		Seed of the 1948 Harvest at 2° C.		
0	Not determined	4·106	0·0124	4·118
3	,,	3·932	0·0144	3·952
6	,,	3·770	0·1210	3·891
9*	,,	3·440	0·3330	3·773
12	,,	2·905	0·7900	3·693
15	,,	1·976	1·5500	3·526
		Seed of the 1948 Harvest at 15° C.		
0	Not determined	4·106	0·0124	4·118
3	,,	3·951	0·0140	3·965
6	,,	4·000	0·0400	4·040
9	,,	4·500	0·0338	4·554
12	,,	3·540	0·0240	3·564
15	3·529	3·489	0·0310	3·520
24	3·393	3·379	0·0307	3·410

Weighings were made accurately to 0·05 mg. Since the samples weighed contained about 250 embryos, the weights expressed as mg. per embryo, though small, do not imply too high a degree of accuracy.

It will be noticed that the dry weight of endosperm plus embryo is almost invariably greater than that of the whole seed. This is explained by the fact that any sample of whole seed includes a number without embryos; when embryos are dissected out and separate weighings of endosperm and embryo are made, only perfect seeds are included.

* The end of the necessary low-temperature treatment occurs at 9 weeks and the seeds germinate at 12 weeks.

Dry weight (cf. Table I)

Variability in samples in most cases masks any systematic change in seed dry weight with time, which is naturally expected, but the loss is estimated to be probably less than 10 per cent.

Changes in the carbohydrate fraction (see Appendix, Tables II and III)

Only simple sugars were estimated quantitatively. In no case was any non-fermentable reducing substance found to be present. All results are therefore given as equivalent to mg. of glucose.

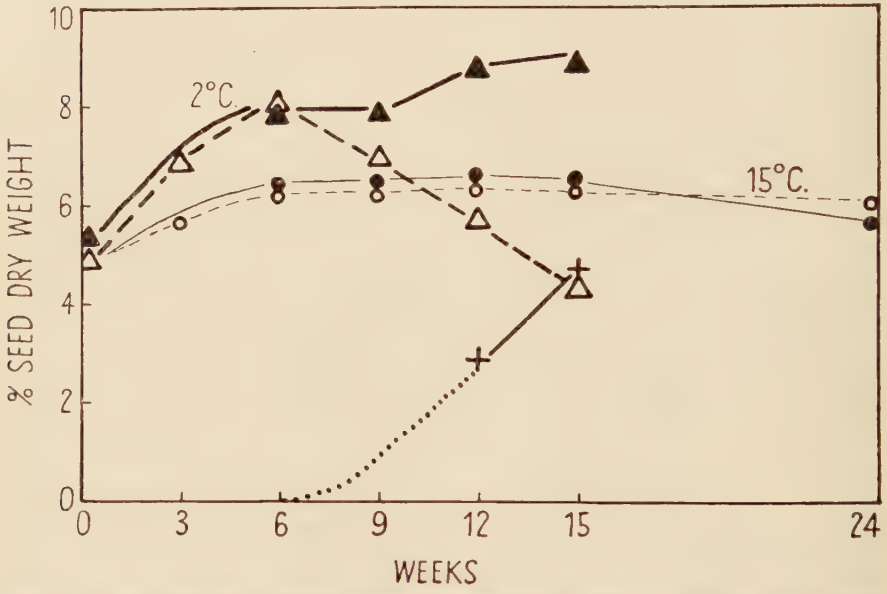

FIG. 1. Sugar contents during treatment expressed as a percentage of seed dry weight. ▲——▲ whole seed content; Δ- - - -Δ endosperm content; +——+ embryo content at 2° C.; ●——● whole seed content; O- - - -O endosperm content at 15° C.

The soluble sugar content of the seeds rises from 5 to 8 per cent. during the first 6 weeks at 2° C. (see Fig. 1). From then on there is a gradual increase in soluble sugars in the seed as a whole, but a progressively larger fraction is located in the embryo. At 15° C. there is a similar rise of approximately 2 per cent. soluble sugar in the whole seed during the first 6 weeks. This level then remains constant until after 15 weeks, when there is possibly a slight decrease. There is no transference of sugar to the embryo detectable from the differences between whole seed and endosperm contents.

Starch and dextrins are absent from the seed initially, but as development proceeds at 2° C., dextrins are formed in progressively greater amounts in the embryo until, after 6 weeks, starch begins to appear in the region of the hypocotyl. This spreads until by the end of after-ripening the embryo is full of starch (see Fig. 2). After germination, however, the starch begins to disappear again.

No starch was found in the endosperm at all, though soon after germination dextrins appeared in the ruptured edges. The cell walls, however, are very thick and it is possible that polysaccharides and hemicelluloses are acting as storage carbohydrates. During low-temperature treatment the cell walls become very much thinner and finally disintegrate altogether. This provides a possible source for other carbohydrates found to appear at low temperature.

At 15° C. starch and dextrins appear in an early stage in the embryo. The presence of starch follows the accumulation of dextrins in time, but there is

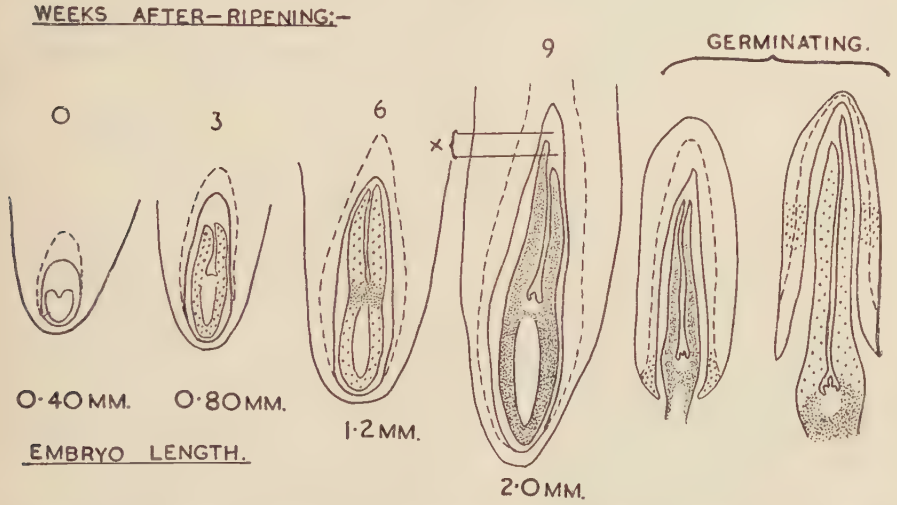

Fig. 2. Development of embryo of *Heracleum sphondylium* within the cavity of the endosperm at 2° C. The dotted line marks the zone of disintegrating cells around the cavity; wide stipling denotes the distribution of dextrins; close stipling denotes the distribution of starch. N.B. Diagrams of embryos during after-ripening are on the same relative scale. Diagrams of germinating seeds are on a reduced scale.

no correlation between the appearance of starch and the stage of development, as there is at 2° C. Thus at room temperature small embryos contain starch at a stage considerably before that in which starch appears at 2° C. It must be assumed that some sugars are transferred to the embryo, for storage compounds could only be formed with hexose sugars as an intermediate step, and since they are immediately converted into storage form there is presumably more sugar entering the embryo than can be utilized at this temperature.

Changes in the nitrogen fractions (Figs. 3 and 4; Appendix, Tables IV–IX).

At room temperature the changes in the composition of the nitrogenous fractions are negligible. It appears, however, that a very small amount of nitrogen is slowly transferred to the embryo.

At 2° C., however, there is an immediate and rapid breakdown of protein into soluble compounds. During the first 6 weeks 18 per cent. of the insoluble protein fraction of the seed is converted into soluble form (cf. Fig. 3 A). After

this time the overall composition of the nitrogen fractions of the seed, as a whole, remains approximately constant, but in the endosperm the breakdown of protein continues at the initial rate (cf. Fig. 3 B). The soluble nitrogen so

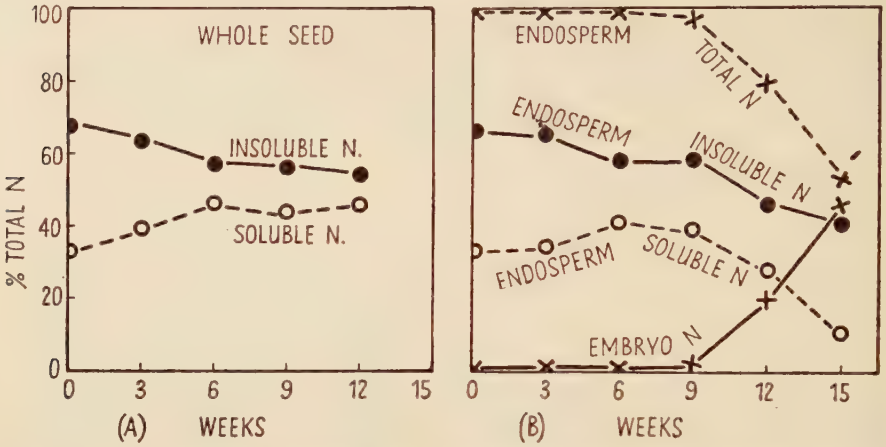

FIG. 3 A. Soluble and insoluble seed nitrogen contents during treatment at 2° C. Expressed as a percentage of total N (*A*) (see Appendix). O - - - O soluble N; ●——● insoluble N.

B. Endosperm and embryo nitrogen contents during treatment at 2° C. expressed as a percentage of total seed nitrogen (*B*) (see Appendix). ●——● insoluble endosperm N; O - - - O soluble endosperm N; X - - - X endosperm total N (soluble+insoluble); X——X embryo total N.

There was no change in the composition of the nitrogen fractions at 15° C.

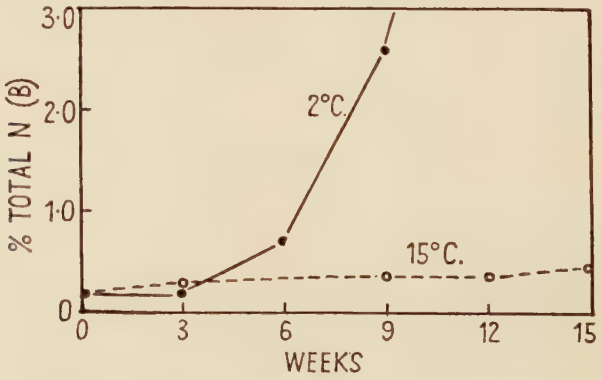

FIG. 4. Embryo nitrogen expressed as a percentage of total seed nitrogen (*B*) (see Appendix).

formed is then transferred into the embryo where it is again converted into an insoluble form, so maintaining the ratio of soluble to insoluble nitrogen in the seed as a whole.

Microscopical examination of sections shows that proteins are stored in the endosperm in the form of granules and it is interesting to note that these are by no means exhausted during the after-ripening process.

Changes in fat content (cf. Fig. 5; Appendix, Table X)

One-third of the seed dry weight is accounted for by ether-soluble substances classified as fats.

At 2° C. no change in fat content is detectable until after the completion of after-ripening, when there is a loss of fats during germination. It should be noted that this result does not agree with the reports of Eckerson (1913) and Pack (1921) that there is a decrease in fat content towards the end of after-ripening. These accounts are, however, based on analyses of seeds after-ripening and germinating at 5° C., so that the failure to distinguish the end

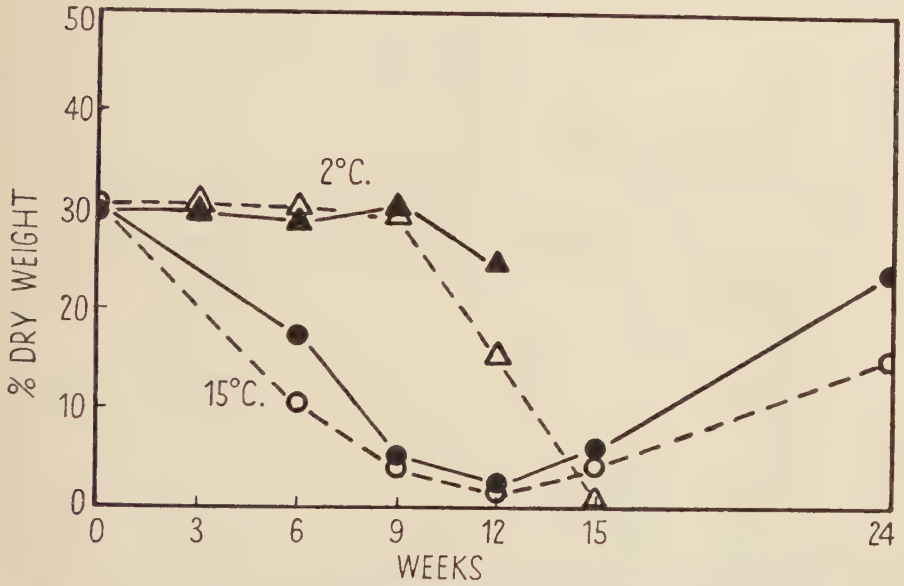

Fig. 5. Fat content of seeds and endosperms expressed as a percentage of seed dry weight. ▲——▲ whole seed content; Δ - - - Δ endosperm content at 2° C.; ●——● whole seed content; O - - - O endosperm content at 15° C.

of the after-ripening process and the commencement of germination may account for the apparent discrepancy.

At 15° C. there was a marked fall in ether-soluble material, followed by an equally marked rise after 12 weeks. The hypothesis that fats are being rapidly respired at room temperature is doubtful since (1) there is little loss of seed dry weight, (2) no great increase in soluble sugar content, and since (3) long-chain products of partial breakdown would themselves be extractable in ether. Moreover, the improbability that fats would be resynthesized in a predominantly storage tissue throws considerable doubt upon the reliability of the ether extraction method for showing the real fat content. It is, however, recognized that in some cases fats may be combined in the cells so as to render them insoluble in ether without previous saponification (Milner, 1948, and Collyer, private communication). Such a change in the

distribution of fats at room temperature would account for the incomplete extraction.

Changes in acid content (Fig. 6; Appendix, Table XI)

There is a greater acidity at 2° C. than at 15° C. and microscopical examination with the use of indicators shows that a zone of lowest pH comes in the mucilage and disintegrating cells around the embryo (see Fig. 6)

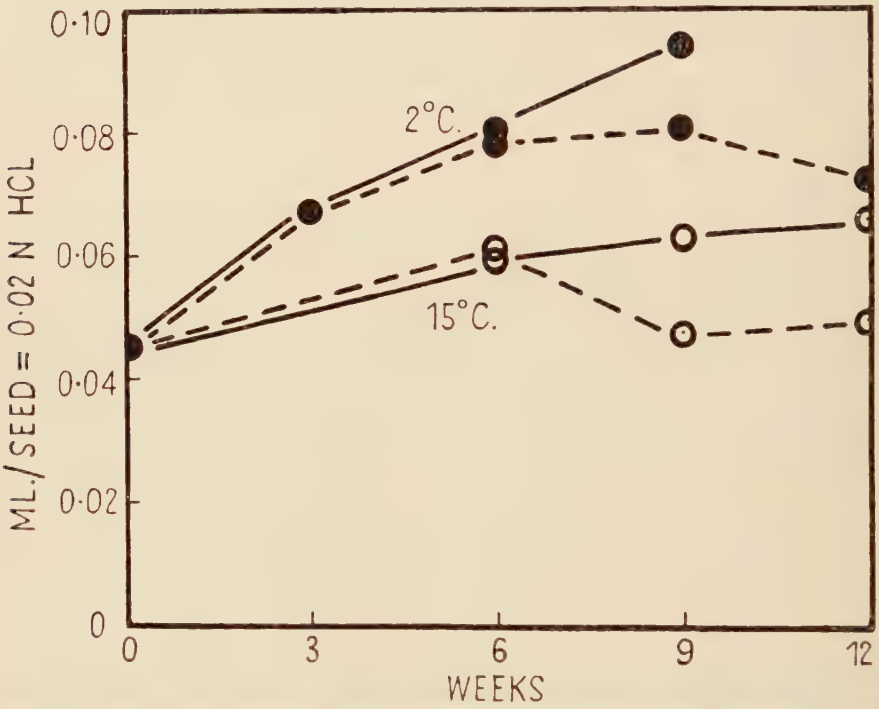

Fig. 6. Acid content of seeds and endosperms expressed as ml./seed equivalent to 0·02 NHCl. ●——○ seed content; ●- - - -○ endosperm content.

Water content (Fig. 7)

Percentage water content of whole seeds is shown in Fig. 7. There is a rapid increase at 2° C. after 6 weeks, but this can hardly reflect the conditions in the embryo itself since the embryo is so very small in the early stages of development.

Discussion

Like other seeds known to require after-ripening, *Heracleum* seeds have a composition of approximately 36 per cent. protein, 30 per cent. fats, and 5 per cent. sugars, but no starch reserves. As the endosperm cell walls disintegrate during after-ripening it is probable that wall hemicelluloses and polysaccharides function as carbohydrate reserves.

At 2° C. there is an immediate and rapid breakdown of endosperm protein to soluble nitrogenous compounds which are rapidly transferred to the embryo, and, together with soluble sugars, utilized for rapid embryo growth. Surplus sugars are stored as starch and dextrins in the embryo in the later stages of development, and an area of low pH develops in the mucilage surrounding the embryo. Fats are utilized in large quantities only during germination.

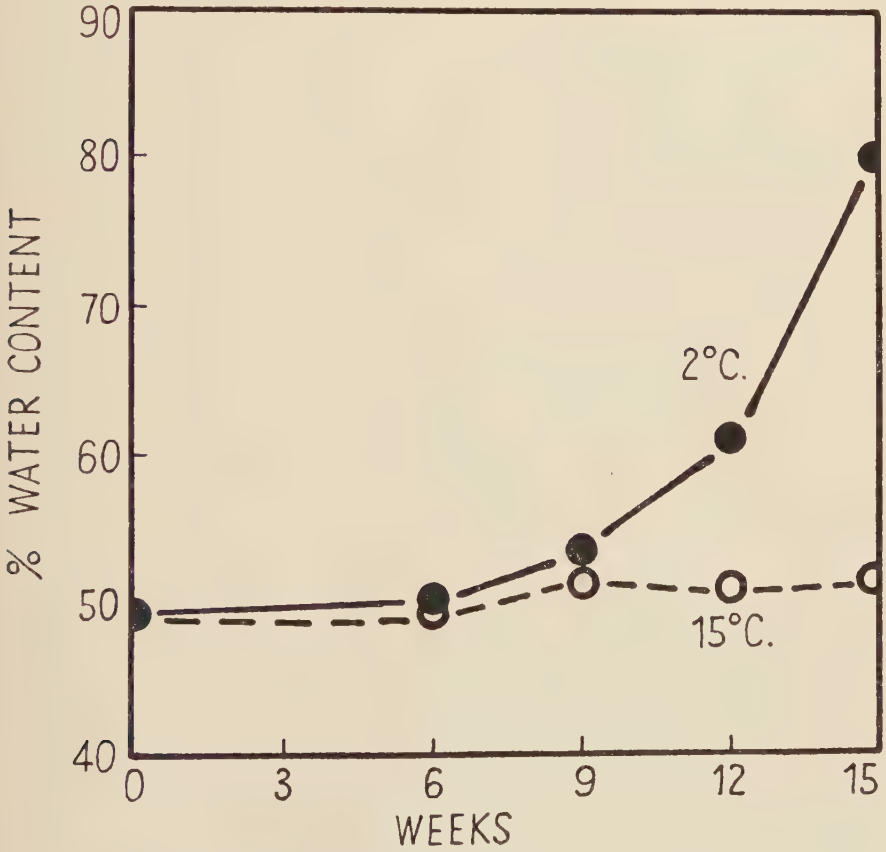

Fig. 7. Percentage water content of seeds at 2° C. and 15° C.

At 15° C., however, starch and dextrins become apparent in the embryo at an early stage. This shows that small quantities of sugars are being transferred to the embryo from the endosperm, and therefore suggests that the cessation of growth at 15° C. is not due to carbohydrate starvation.

The transfer of nitrogenous substances, however, is very slow at 15° C. and there is virtually no breakdown of endosperm protein at this temperature. Furthermore, the curves for embryo nitrogen content (Fig. 4) and the growth curves obtained for the two temperatures are strikingly similar (Stokes, 1952 *a*

Fig. 2). It thus appears that nitrogen starvation is responsible for the cessation of embryo growth at room temperature. The lack of available nitrogenous compounds would also prevent the utilization of available sugars for growth.

The effect of after-ripening on embryo growth depends, therefore, on the action of low temperature in promoting protein breakdown, and the possible mechanism of this may be discussed.

The bulk of the endosperm tissue is composed of thick-walled cells containing dense protoplasm, abundant protein granules, and fat droplets. As the embryo develops the cells around the cavity gradually lose their contents and disintegrate. The protein granules are still abundant in the outer cells of the endosperm, but proteins are entirely absent from the disintegrating cells around the embryo cavity. There is, in fact, a gradient of decomposition radiating outwards from the embryo, which suggests that low temperature acts not directly on the proteins themselves but via the embryo.

This suggestion is further substantiated by the fact that during the period of low-temperature treatment necessary for after-ripening, only some 20 per cent. of the endosperm protein is broken down. The remainder decomposes during germination without the action of low temperature, provided the embryo has been fully after-ripened; that is to say, made capable of causing protein breakdown in the endosperm.

It is unlikely that this action of the embryo is due to the excretion of enzymes, since it could not be reconciled with the length of low temperature necessary for after-ripening. If the excretion of enzymes were initiated by low temperature, then, once present in the endosperm, they would be expected to continue functioning, and with an increased efficiency, when the seeds are removed to room temperature after, say, 6 weeks. Experiment shows, however, that growth ceases in seeds removed from the cold before the completion of after-ripening (Stokes, 1952*b*).

It is therefore suggested that some product of embryo metabolism at low temperature diffuses into the endosperm, bringing about the breakdown of storage proteins. It has already been shown that an area of low pH develops in the mucilage around the embryo, and it is possible that the excretion of acids at low temperature could bring about the changes described. A critical concentration, reached after 9 weeks at low temperature, would then enable breakdown to proceed at room temperature without further replenishment of acids. The probability of this suggested mechanism is further substantiated by experiments of Eckerson (1913) in which after-ripening of *Crataegus* seeds was accelerated by treatment with dilute acids.

Conclusion

Products of embryo metabolism at low temperature initiate the breakdown of endosperm storage protein into soluble compounds available for embryo growth. At room temperature growth is prevented by nitrogen starvation.

Summary

1. The effect of temperature on seed metabolism was investigated at 2° C. and 15° C. at 3-weekly intervals from 0 to 15 weeks at 2° C. and after 24 weeks at 15° C.

2. The changes in the following fractions of seeds and endosperms were estimated quantitatively: soluble and insoluble nitrogen content, sugar content, fat content, titratable acidity, and water content.

Total embryo nitrogen was estimated quantitatively, as was sugar content where possible.

Microscopical examination was made for the distribution of reserve carbohydrates, proteins, and acids.

3. At low temperature there is a rapid conversion of protein into soluble nitrogenous compounds which, together with soluble sugars, enables growth and germination of embryos after 9 weeks at 2° C.

4. The possible action of low temperature in the breakdown of proteins is discussed.

Acknowledgement

I am indebted to Professor M. Skene, under whose supervision this work was carried out in the Botany Department of the University of Bristol, and to Dr. E. W. Yemm for his advice on the analytical techniques. I also wish to express my appreciation of a grant from the Agricultural Research Council which enabled the work to be undertaken.

Literature Cited

Baker, J. R., 1947: The Histochemical Recognition of Certain Guanidine Derivatives. Quart. J. Micro. Sci., 3rd ser., lxxxviii. 115–21.

Chibnall, A. C., Rees, M. W., and Williams, E. F., 1943: The Total Nitrogen Content of Egg Albumin and Other Proteins. Biochem. J., xxxvii. 354–9.

Eckerson, S., 1913: A Physiological and Chemical Study of After-ripening. Bot. Gaz., lv. 286–99.

Milner, H. W., 1948: The Fatty Acids of Chlorella. J. Biol. Chem., clxxvi. 813–17.

Pack, D. A., 1921: Chemistry of After-ripening and Germination and Seedling Development of Juniper Seeds. Bot. Gaz., lxxii. 139–50.

Somogyi, M., 1945; A New Reagent for the Determination of Sugars. J. Biol. Chem., clx. 61–68.

Stokes, Pearl, 1952 *a*: A Physiological Study of Embryo Development in *Heracleum sphondylium* L. I. Effect of Temperature on Embryo Development. Ann. Bot., N.S.

—— 1952 *b*: II. Effect of Temperature on After-ripening. Ann. Bot., N.S.

Vaneslow, A. P., 1940: Preparation of Nessler's Reagent. Annal. Ind. Eng. Chem., xii. 516–17.

Appendix

ANALYTICAL RESULTS

Results are given both as mg. per seed and as percentage of dry weight. The fluctuations in the seed dry weight already referred to do not mask the general drifts shown but occasionally lead to figures which seem aberrant.

SUGAR CONTENT

Mean differences between duplicate determinations of sugar estimations are 2·1±0·75 per cent.

Table II

Sugar in Seed, Endosperm, and Embryo, in mg. per Seed

	Weeks.	Seed 1947.	Endosperm 1947.	Endosperm 1948.	Embryo 1948.
At 2° C.:	0	0·2060	0·1848	0·1960	—
	3	—	0·2949	0·2572	—
	6	0·2810	0·3236	0·3471	—
	9	0·3045	0·2557	0·2730	—
	12	0·3125	0·2733	—	0·1041
	15	—	—	0·1520	0·1631
At 15° C.:	0	0·2060	0·1798	0·1960	
	3	0·2323	—	0·2245	
	6	0·2477	0·2469	0·2503	
	9	0·2527	0·2152	—	
	12	0·2297	0·2411	—	
	15	0·2594	—	0·2252	
	24	0·1917	—	0·2085	

Table III

Sugars expressed as Percentage of Seed Dry Weight

	Weeks.	Seed 1947.	Endosperm 1947.	Endosperm 1948.	Embryo 1948.	Endosperm+ embryo 1948.
At 2° C.:	0	5·4	4·6	4·8	—	—
	3	—	7·6	6·5	—	—
	6	7·9	8·0	8·1	—	—
	9	7·9	6·5	7·2	—	—
	12	8·8	5·7	—	2·9	8·5
	15	8·9	—	4·3	4·6	8·9
At 15° C.:	0	5·4	4·6	4·8		
	3	—	—	5·7		
	6	6·5	6·6	6·2		
	9	6·8	6·3	—		
	12	6·7	6·5	—		
	15	6·6	—	6·4		
	24	5·7	—	6·1		

NITROGEN FRACTIONS

Mean difference between duplicates of all nitrogen (N) determinations (except embryos) is 3·5±0·71 per cent.

In Tables IV and V the values are derived as follows:

a. Determination of total nitrogen in whole seed, i.e. Total N (*A*).

b. Sum of determinations of soluble and insoluble nitrogen in endosperm and of total nitrogen in embryo, i.e. Total N (*B*).

c. Sum of determinations of soluble and insoluble nitrogen in whole seed, i.e. Total N (*C*).

TABLE IV

Total Nitrogen found by Different Methods as Mg. per Seed

	Weeks.	0.	3.	6.	9.	12.	15.	24.
At 2° C.:	*A*	0·1917	0·2029	0·1946	0·2145	0·2052	—	—
	B	0·2243	0·2400	0·2394	0·2175	0·2340	0·2135	—
	C	0·2187	0·2079	0·2017	0·2100	—	—	—
At 15° C.:	*A*	0·1917	—	0·1932	0·1996	0·2040	0·2099	0·2050
	B	0·2223	—	—	0·1947	0·2034	0·2071	0·1703
	C	0·2187	—	0·2114	0·1996	—	0·1665	0·1989

TABLE V

Total Nitrogen found by Different Methods as Percentage of Seed Dry Weight

	Weeks.	0.	3.	6.	9.	12.	15.	24.
At 2° C.:	*A*	5·02	5·57	5·46	5·57	5·80	—	—
	B	6·05	6·58	6·73	5·65	6·60	6·05	—
	C	5·72	5·69	5·66	5·33	—	—	—
At 15° C.:	*A*	5·02	—	5·58	5·54	5·75	5·95	6·05
	B	6·05	—	—	5·41	5·35	5·87	5·00
	C	5·72	—	5·89	5·00	—	4·71	5·84

(In Table V series *A* and *C* are expressed as percentage whole seed dry weight, *B* is expressed as percentage of dry weight of endosperm plus embryo. Cf. Table I.)

There is no significant decrease in Total N (*A*) content throughout the analyses, the values lying between 5 and 6 per cent. of seed dry weight.

Total N as calculated by the addition of soluble and insoluble endosperm N and embryo total N, i.e. Total N (*B*), gives results on an average 1 per cent. of dry weight higher than total N (*A*) determined directly. As in the case of dry weights, this is due to there being no means of checking that all whole seeds analysed were good seeds as was possible with those which were dissected.

Recovery of seed nitrogen in the soluble and insoluble fractions, i.e. total N (*C*), comes within 5 per cent. of the total N (*A*) in most cases.

TABLE VI

Insoluble, Soluble, and Total Nitrogen as Mg. per Seed

		Seed			Endosperm		
	Weeks.	Insol. N.	Sol. N.	Total N. (insol. + sol. N.)	Insol. N.	Sol. N.	Total N. (insol. + sol. N.)
At 2° C.:	0	0·1363	0·0824	0·2187	0·1487	0·0758	0·2240
	3	0·1267	0·0812	0·2079	0·1510	0·0835	0·2395
	6	0·1120	0·0897	0·2017	0·1390	0·0987	0·2377
	9	0·1210	0·0890	0·2100	0·1266	0·0838	0·2160
	12	—	0·0945	—	0·1083	0·0669	0·1751
	15*	—	—	—	0·0894	0·0250	0·1164

TABLE VI (*cont.*)

		Seed			Endosperm		
	Weeks.	Insol. N.	Sol. N.	Total N. (insol.+ sol. N.)	Insol. N.	Sol. N.	Total N. (insol.+ sol. N.)
At 15° C.:	0	0·1363	0·0824	0·2187	0·1563	0·0681	0·2244
	3	—	—	—	0·1330	0·0606	0·1936
	6	0·1348	0·0706	0·2114	0·1524	0·0735	0·2259
	9	0·1348	0·0648	0·1996	0·1295	0·0628	0·2923
	12	—	0·0643	—	0·1348	0·0677	0·2025
	15	0·1166	0·0499	0·1665	0·1515	0·0577	0·2193
	24*	0·1349	0·0640	0·1989	0·1205	0·0486	0·1693

TABLE VII

Insoluble and Soluble Nitrogen expressed as Percentage Total N

		Seed (% Total N (*A*))		Endosperm (% Total N (*B*))		
	Weeks.	Insoluble.	Soluble.	Insoluble.	Soluble.	Total.
At 2° C.:	0	67·6	33·0	66·3	33·7	99·98
	3	63·5	39·0	65·0	34·8	99·81
	6	57·0	46·0	58·0	41·3	99·28
	9	56·5	43·5	58·3	39·0	97·61
	12	54·4	45·6	46·0	28·5	79·87
	15*	—	—	41·7	11·7	53·7
At 15° C.:	0	67·6	33·0	66·3	33·7	99·98
	3	—	—	68·25	31·5	99·97
	6	67·5	35·5	67·6	32·3	99·9
	9	68·5	32·5	66·7	32·3	99·64
	12	—	31·3	66·3	33·3	99·64
	15	70·0	30·0	73·5	28·0	99·55
	24*	65·8	31·2	70·8	28·3	99·28

TABLE VIII

Total Nitrogen of the Embryo expressed as Percentage Total Seed N (*B*)

	2° C.		15° C.	
Weeks.	1947.	1948.	1947.	1948.
0	0·115	—	0·115	—
3	0·196	—	0·255	—
6	0·72	—	—	—
9	2·62	—	0·36	—
12	20·13	—	0·36	—
15	—	46·30	0·45	—
24	—	—	—	0·72

(Only one set of results for each sample is expressed as percentage total seed nitrogen as only one sample had an analysis done on both seed and embryo.)

* Indicates those analyses made on 1948 seed. All the others were made on 1947 seed.

TABLE IX

Total Nitrogen of the Embryo as Mg. per Seed

Weeks.	2° C. 1947.	2° C. 1948.	15° C. 1947.	15° C. 1948.
0	0·00026	0·00044	0·00026	0·00044
3	0·00047	0·00047	—	0·00040
6	0·00173	0·00210	0·00022	0·00055
9	0·0057	0·0014	0·00071	0·00094
12	0·0590	0·0180	0·00074	0·00149
15	—	0·0995	0·00093	0·00101
24	—	—	—	0·00123

(As has been previously explained, there was insufficient embryo material to make duplicate determinations on any one sample. The whole series of analyses was therefore repeated on seeds from the next year's harvest and although the actual weights differ considerably, the essential changes are repeated in the second year seed.)

Embryo N content is shown in Fig. 5 as percentage total N (*B*). Such small quantities, whilst they are highly significant so far as the embryo itself is concerned, are very insignificant when compared with total seed N.

FAT CONTENT

TABLE X

Fat Content of Seeds and Endosperms as a Percentage of Seed Dry Weight

Weeks.	2° C. Seed % Dry weight (*A*).	2° C. Endosperm % Dry weight (*B*).	15° C. Seed % Dry weight (*A*).	15° C. Endosperm % Dry weight (*B*).
0	29·5	30·0	29·5	30·0
3	29·5	29·1	—	—
6	28·5	31·4	17·1	10·6
9	30·4	29·5	5·35	4·1
12	24·2	15·9	2·6	1·9
15	—	1·7	6·1	4·3
24	—	—	23·2	15·0

ACID CONTENT

TABLE XI

Acid Content in ml. equivalent to 0·02 NHCl *per seed*

	Dormant.	3.	6.	9.	12.
At 2° C.: Seed 1947 .	0·0462	—	0·0801	0·0940	—
Endosperm 1947	0·0462	0·0675	0·0780	0·0810	0·0729
At 15° C.: Seed 1947 .	0·0462	—	0·0600	0·0630	0·0660
Endosperm 1947	0·0462	—	0·0610	0·0470	0·0490

Observations on the Formation and Function of the Root Nodules of *Alnus glutinosa* (L.) Gaertn.

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With Plate III and one Figure in the Text

Abstract

Nodulated alder plants grow vigorously in water culture without combined nitrogen. Evidence is advanced to show that the fixation of atmospheric nitrogen thus implied occurs actually within the nodulated plant and probably in the nodule.

Nodule formation occurred most freely over the pH range 5·4 to 7·0, while subsequent to nodulation the best growth of plants was in the pH range 4·2 to 5·4. The capacity of the host plant to tolerate relatively low pH levels considerably exceeds that of the nodule organism. The oxygen requirement of the nodules appears to be relatively high. The fixation of nitrogen per unit dry weight of nodule tissue exceeds that of legumes grown under comparable conditions.

Introduction

THERE is considerable evidence in the literature that the ability to fix atmospheric nitrogen is associated with the nodules normally present on the root system of the alder. Previous observations relating to this feature in *Alnus* species are to be found in the papers of Hiltner (1896), Nobbe and Hiltner (1904), Möller (1912), Krebber (1932), Roberg (1934), Virtanen and Saastamoinen (1936), and Plotho (1941). In addition a recent paper by Hawker and Fraymouth (1951) will be considered. Except for those of Virtanen and Saastamoinen, these observations referred essentially to the primary question of whether fixation of nitrogen was associated with nodulated plants, and since in each instance it was found that the nodulated alder grew vigorously in water

[Annals of Botany, N.S. Vol. XVII, No. 65, January, 1953.]

or sand culture free of combined nitrogen, all the authors drew a justifiably affirmative conclusion. Nobbe and Hiltner grew their plants to an age of 8 years, by which time a height of 2 metres had been attained, while in Möller's work analyses were included which confirmed that large amounts of nitrogen (5·6 g. per plant) had been accumulated.

Virtanen and Saastamoinen (loc. cit.) in addition studied the effect of pH on the growth of nodulated plants which had already grown for two seasons under uniform conditions. The pH of the rooting medium (sand) was then adjusted to different values and the subsequent growth of the plants observed over a further two seasons. It was concluded that pH 6 was the most favourable level for nodulated alders, and that the same applied to non-nodulated plants supplied with ammonium nitrate. These findings must be regarded as being of tentative nature, since data were obtained for only a few plants at each pH level.

The present studies have proceeded along the following lines:

(1) the effect of pH on the infection and nodulation of alder and on the subsequent growth of nodulated plants has been examined in water culture, parallel non-nodulated plants supplied with combined nitrogen being included in the experiment in order to enable effects specific for inoculated plants to be distinguished;
(2) the effect of oxygen supply on nodule development and activity has been examined;
(3) using the new data obtained, comparisons have been made between non-legumes and legumes with regard to quantitative aspects of the symbioses.

Hawker and Fraymouth (1951), without actually quoting any references, have suggested that a study of the literature leaves open the question as to whether the endophytes of the root nodules of *Alnus*, *Myrica*, *Hippophaë*, and *Elaeagnus* are able to fix nitrogen. If by this they mean that the literature is equivocal as regards the association of a power of nitrogen fixation with nodulated plants of these genera, which is the only significant interpretation that can be placed upon their statement, then to the present authors this appears to be an incorrect assessment of earlier work, certainly as regards the first two genera. Good evidence for fixation in *Myrica* has been provided by Bond (1949, 1951*b*), while as has been indicated already all the previous investigations relating to *Alnus* known to the present authors have given positive indication of fixation, and though it is always possible that they have overlooked an odd paper with contrary findings, there is clearly a strong body of positive evidence which cannot be set aside.

In the same paper Hawker and Fraymouth refer very briefly to their own preliminary experiments in which nodulated alders in garden soil were found to grow no better than plants without nodules, this being apparently interpreted as indicating that no faculty for fixation of nitrogen was associated with the former plants. It is to be hoped that the authors mentioned will see

whether their result can be confirmed when a rooting medium permitting control over the access of combined nitrogen to the plants is used. Meanwhile in view of Hawker and Fraymouth's paper the further evidence for fixation obtained incidentally in the present work will be presented in somewhat greater detail than would otherwise have been necessary.

Methods

Seed of *Alnus glutinosa* was sown in early spring in horticultural peat, neither the seeds nor the peat receiving any sterilizing treatment. Seedlings were transplanted into water culture about 8 weeks from sowing, by which time they were about 1·5 cm. high and bore one to two leaves. No nodules were present. In most of the work glazed earthenware jars of 2-litre capacity were used for the water cultures, each being covered by a waxed square of teak with 7 holes in which the plants were fixed. The basic culture solution was a nitrogen-free form of Crone's solution (plus minor elements) prepared as previously specified (Bond, 1951*b*). The pH of this solution was adjusted as required by addition of sulphuric acid or sodium hydroxide.

The inoculation of plants with the nodule organism was effected on the day following transplanting by applying to the root system a drop of a suspension in water of crushed nodules from alders in the field. Autoclaved inoculum was applied to control plants.

At harvest, dry weights of individual plants were obtained by heating at 95° C. and total nitrogen was estimated by the Kjeldahl process.

Data

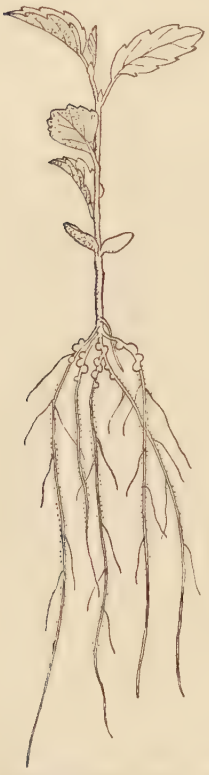
Effect of pH on nodulation and growth of plants

The main experiment on this aspect was carried out in 1951 and comprised initially 63 jars with inoculated plants in nitrogen-free solution and uninoculated plants, some in nitrogen-free solution and others supplied with combined nitrogen in the form of sodium nitrate or ammonium sulphate. In each of these categories plants were set up at five pH levels over the range 3·3 to 7·0. There was no forced aeration of the cultures.

In the case of the *inoculated plants* nodules became visible to the naked eye 2 to 3 weeks after inoculation, when the young plants had formed 3 to 4 leaves and were still only 2 to 3 cm. in height (see Text-fig. 1). With earlier inoculation nodules could probably have been obtained at a still younger stage in plant development. Thus in alder, nodules form at much the same stage in host-plant development and after a similar interval from the time of inoculation as in legumes growing under comparable conditions. The same is true of *Myrica gale* (Bond, 1951*b*).

The young alder nodules were mostly light brown in colour on plants growing in earthenware jars, but in glass containers they were intensely red in colour, possibly because the access of light to the root system promotes the

development of the red pigmentation. A similar nodule colour was also noted by Krebber (1932) in alder and by Bond (1951*b*) in *Myrica gale* and was shown in the latter to be due to anthocyanin.



Young alder plant with root nodules, from water culture, 32 days after inoculation. In this particular plant nodules were first visible to the naked eye 12 days from inoculation. (Nat. size.)

Nodules appearing in response to the normal inoculation effected shortly after transplanting into water culture were, in these non-aerated cultures, mostly situated high up on the root system near to the base of the hypocotyl (see Text-fig. 1, also Pl. III, Fig. 1), but those induced by a second inoculation which, as explained below, was applied a month later to the plants of the main experiment, were in most cases at lower levels and well submerged in the culture solution (Pl. III, Fig. 2). This suggests that the location of the nodules is affected by the stage of development of the root system at the time of inoculation, particularly, perhaps, by the distribution of root-hairs.

Branching of the originally simple nodules soon occurred, leading to the formation of nodule clusters. No development of roots from the nodules as occurs in *Myrica gale* (Bond, 1952) has been observed in alder. Peculiar flattened conical projections frequently present on the nodules proved to be the hypertrophied complementary tissue of the lenticels which, as noted by Borm (1931), normally occur on the nodules.

The initiation of nodules was considerably affected by the pH of the surrounding culture solution, as shown in Table I. There was a complete failure of nodulation in the most acid solution, while at pH 4·2 the proportion of plants forming nodules was small. Of the pH levels employed, 7·0 appears to be the most favourable for inoculation, but tests with larger numbers of plants would be desirable to confirm that this pH is really more favourable than pH 6·3 or 5·4.

Indications of the commencement of fixation of nitrogen were first noted 2 weeks after the first appearance of nodules, the growth of nodulated plants beginning then to surpass that of the uninoculated control plants in nitrogen-free solution. From this time onwards the nodulated plants grew with increasing vigour, and after 3 months' growth it was necessary to reduce the number of plants to 3 per jar.

As the plants became larger it also became necessary to readjust the pH of the culture solution in the jars every day, in order to counteract the tendency for a fall to occur as the result of root action. During the period of most active growth the pH fell by about 0·4 of a unit during 24 hours.

TABLE I

Effect of pH of Culture Solution on Nodulation

pH of culture solution.	Number of plants forming nodules	
	1950 expt.	1951 expt.
3·3	0/12*	0/35*
4·2	0/12	7/35
5·4	2/12	23/35
6·3	6/12	22/35
7·0	—	28/35

* In 1950 the total number of plants inoculated at each pH was 12 and in 1951 was 35.

In Table II the data obtained at the harvest of the nodulated plants of the 1951 experiment, after 6 to 7 months' growth in water culture, are presented together with statistical treatment, the plants being placed in two groups for reasons explained in the table. The smaller number of plants available at the lower pH levels is, of course, a reflection of the inferior success of inoculation under these conditions, as noted already. Plants which failed to nodulate soon became moribund and were discarded prior to harvest. Very considerable plant-to-plant variation was shown at a given pH level, so that relatively large differences in means are required for significance.

The general sequence of the data in Table II is the same for both groups of plants, the highest mean figures for height, dry weight, and nitrogen content being shown at pH 5·4. Reference to the statistical treatment shows that (*a*) the superiority of growth at pH 5·4 over that at higher pH levels is fully significant in almost all respects, (*b*) there is no significant difference between growth at pH 6·3 and 7·0, and (*c*) the apparent superiority of growth at pH 5·4 over that at 4·2 fails to attain significance. The leaf colour was uniformly dark green at all pH levels. Typical plants from the second group are shown in Pl. III, Fig. 3.

Data for the *uninoculated control plants* in nitrogen-free solution, all of which remained free of nodules, are shown in Table III. The inability of these plants to make any appreciable growth confirms that no unsuspected sources of combined nitrogen were provided by the experimental arrangement. It is therefore concluded that practically all the nitrogen accumulated by the nodulated plants (Table II) was the result of fixation of atmospheric nitrogen. It will be noted that at pH 5·4 the mean fixation in the first group of plants over the 6-month growth period was 301 mg. per plant, the highest individual value being 356 mg. In Pl. III, Fig. 4, typical nodulated and control plants are compared.

Turning to the *uninoculated plants supplied with combined nitrogen*, the results obtained with sodium nitrate as the source of nitrogen will be considered first. Daily adjustment of pH was again necessary. At the three lower pH levels the value tended to rise, and at times of rapid growth an increase of up to 0·7 was shown over 24 hours in the nominally pH 3·3 series. At no stage

TABLE II

*Mean Data obtained at Harvest of Nodulated Plants grown in Nitrogen-free Solution at Different pH Levels**

	pH of culture solution.	No. of plants harvested.	Height of shoot (cm.).	Dry weight per plant (g.)			Total nitrogen per plant (mg.).
				Nodules.	Top and root.	Total.	
1st group	3·3	0	—	—	—	—	—
	4·2	2	38	0·152	10·60	10·75	153
	5·4	8	52	0·370	16·80	17·17	301
	6·3	5	41	0·231	9·21	9·44	170
	7·0	13	38	0·246	11·68	11·93	232
2nd group	3·3	0	—	—	—	—	—
	4·2	5	21	0·155	3·58	3·74	86
	5·4	15	23	0·209	4·29	4·50	100
	6·3	17	11	0·062	1·43	1·49	34
	7·0	15	11	0·066	1·48	1·55	37

* Seedlings were transplanted into water culture on April 11, 1951, with 35 at each pH. The plants of the 1st group formed nodules in response to the original inoculation and were harvested Oct. 15, 1951. The plants of the 2nd group nodulated after a re-inoculation effected on May 18 and were harvested Nov. 13, 1951.

Minimum Differences between Means required for Significance at $P = 0{\cdot}05$, from Analysis of Variance

	Comparison.	Height of shoot (cm.)		Total dry weight (g.)		Total nitrogen (mg.).	
		Reqd.	Obsvd.	Reqd.	Obsvd.	Reqd.	Obsvd.
1st group	pH 5·4 and 6·3	12	11	5·87	7·73	112	131
	pH 5·4 ,, 7·0	9	14	4·62	5·24	89	68
	pH 6·3 ,, 7·0	11	3	5·36	2·49	102	63
2nd group	pH 4·2 ,, 5·4	4	2	1·29	0·76	30	14
	pH 5·4 ,, 6·3	3	12	0·91	3·01	21	66
	pH 6·3 ,, 7·0	3	0	0·91	0·05	21	3

TABLE III

*Mean Data obtained at Harvest of Non-nodulated Control Plants in Nitrogen-free Solution**

pH of culture solution.	No. of plants harvested.	Height of shoot (cm.).	Dry weight per plant (mg.).	Total nitrogen per plant (mg.).
3·3	5	2·5	18	0·4
4·2	5	2·5	19	0·4
5·4	10	2·5	18	0·4
6·3	7	2·5	21	0·4
7·0	9	2·5	16	0·3

* The plants were set up in water culture on the same date as the inoculated plants (see Table II), with 7 plants at each of the two lowest pH levels and 14 at each of the remaining levels. Some of the plants died at an early stage and were discarded.

in the experiment was there any consistent difference in the growth of the plants at the different pH levels except in respect of height. The harvest data are shown in Table IV, and although the dry weight at pH 5·4 is greater than at other levels, the differences do not attain significance. Height of shoot is, however, significantly greater at that pH than at 3·3 or 7·0.

TABLE IV

*Mean Data obtained at Harvest of Non-nodulated Plants supplied with Nitrate-nitrogen**

pH of culture solution.	No. of plants harvested.	Height of shoot (cm.).	Dry weight per plant (g.).	Total nitrogen per plant (mg.).
3·3	15	54	12·82	163
4·2	15	57	12·53	166
5·4	15	60	13·34	164
6·3	15	55	11·86	163
7·0	15	51	11·09	163

* Seedlings were transplanted into water culture on April 11, 1951. There were initially 5 jars each with 7 plants at each pH, but in July the number of plants per jar was reduced to 3. Harvest was on Sept. 15, 1951, and during the growth period a total of 275 mg. nitrate-nitrogen per plant was added to the culture solution.

Minimum Differences between Means required for Significance at P = 0·05, from Analysis of Variance

Comparison.	Height of shoot (cm.).	Total dry weight (g.).
pH 3·3 and 5·4	Reqd. 5; obsvd. 5	Reqd. 2·44; obsvd. 0·52
pH 4·2 „ 7·0	„ 5; „ 6	„ 2·44; „ 1·44
pH 5·4 „ 7·0	„ 5; „ 9	„ 2·44; „ 1·25

One jar of plants supplied with nitrogen as ammonium sulphate was set up at each pH, the general result being that growth was similarly vigorous at all pH levels except pH 3·3, where it was inferior. It should be noted that, as was to be expected, the pH in these cultures supplied with ammonium-nitrogen tended to fall, so that the plants grown nominally at pH 3·3 were exposed for short periods to pH as low as 2·8.

In the earlier stages of the experiment the plants supplied with combined nitrogen grew more rapidly than the nodulated plants, but this difference disappeared later and by the time of harvest the best of the nodulated plants were superior to the combined nitrogen plants in dry weight and nitrogen content, and had larger, darker-green leaves. Pl. III, Fig. 5, shows plants of each type. In respect of the nodulated plants it may be noted that in the first group the nodule dry matter showed a nitrogen content of 3·58 per cent. compared with 1·79 per cent. for the rest of the plant (mean figures for all pH levels).

The effect of oxygen supply on nodulation and growth

Here again inoculated and uninoculated plants (the latter supplied with combined nitrogen) were grown in water culture, in this instance a uniform

pH of 6·3 being maintained, while half the jars of each type were given forced aeration. Instead of the wide straight-sided jars used for the pH work, jars of similar capacity but of diameter 16 cm. narrowing to 9 cm. at the neck were employed in order to give somewhat greater control over the access of oxygen to the culture solution. The cultures were not made air-tight, and fairly free gaseous diffusion could occur between the external atmosphere and the air-space below the cork in which the plants were fixed. A vigorous current of compressed air was supplied continuously to a proportion of the cultures.

TABLE V

*Mean Data obtained at Harvest of Aeration Experiment**

Type.	No. of plants harvested.	Height of shoot (cm.).	Dry weight per plant (gm.)			Total nitrogen per plant (mg.).
			Nodules.	Top and root.	Total.	
Nodulated, non-aerated	20	11	0·059	1·48	1·54†	36
Nodulated, aerated	18	16	0·126	3·03	3·16	73
Non-nodulated, non-aerated	14	23	—	—	3·16	75
Non-nodulated, aerated	13	19	—	—	2·81	73

* For the nodulated series seedlings were set up in nitrogen-free water culture May 18, 1951, and the plants were harvested Oct. 27, 1951. The corresponding growth period for the non-nodulated plants was from July 13 to Oct. 23. To these latter plants a total of 200 mg. combined nitrogen as ammonium sulphate was supplied per plant.

† The use of the '*t*' test for the dry weight data in the table showed that (at $P = 0{\cdot}05$) the mean for the nodulated aerated plants is significantly greater than that for the corresponding non-aerated plants. The difference in respect of the non-nodulated plants does not reach significance. The same position holds as regards height of shoot.

Nodules soon became more numerous in the aerated inoculated jars and were present at various depths in the solution, whereas in non-aerated jars they were mostly near to the surface. These aerated plants emerged from the nitrogen-hunger period more rapidly, and grew more vigorously throughout the experiment than the corresponding non-aerated plants. In Table V it will be seen that at harvest the mean dry weight and nitrogen content in nodulated plants were doubled as a result of aeration.

In the non-nodulated plants supplied with combined nitrogen growth was essentially unaffected by aeration. The data in Table V actually show a higher mean height and dry weight for the non-aerated plants, but the differences did not attain significance.

The results of determinations of dissolved oxygen in the culture solution, carried out by the syringe-pipette method of Fox and Wingfield (1938), are shown in Table VI and are expressed as a percentage of the oxygen content of water in equilibrium with air at the temperature prevailing at the time of each determination, using the data of Winkler. The results indicate that up to

TABLE VI

Results of Determinations of Dissolved Oxygen in Culture Jars of Aeration Experiment

Type of culture.	Oxygen content of culture solution.*		
	July 12.	Aug. 23.	Oct. 5.
Nodulated, non-aerated	81	53	50
Nodulated, aerated	94	96	97
Non-nodulated, non-aerated	—	51	47
Non-nodulated, aerated	—	93	96

* See text for explanation of method of expression of oxygen data. Each value shown is the mean of 4 jars, the samples being withdrawn from midway down the jars.

the time of the first determinations for nodulated plants (8 weeks after the start of the experiment) the oxygen content in non-aerated jars had fallen only slightly below that in aerated jars, but at later stages it fell to half that maintained by aeration. The initially more rapid growth of the combined nitrogen plants led here to earlier depletion of the oxygen.

DISCUSSION

Evidence for the occurrence of fixation

The finding of earlier investigators that nodulated plants of alder are able to grow vigorously in a rooting medium to which no combined nitrogen has been added is confirmed by the data presented above. The latter refer to plants in their first year of development, but in addition several plants have been grown into a second year, continuously in water culture. One such nodulated plant, from seed sown in April 1950, showed when harvested in October 1951 a height of 112 cm., a dry weight of 219 g., and a total nitrogen content of 2,530 mg., and was in every respect a healthy and vigorous specimen (Pl. III, Fig. 6).

Although fixation of atmospheric nitrogen is obviously associated with the nodulated alder under these conditions, it cannot be stated with finality from the above observations alone that the fixation is actually effected by the nodulated plant itself. This is because the present experiments, like those of all previous investigators, were not of aseptic type, and there is the theoretical possibility that a free-living nitrogen-fixing organism was introduced with the inoculum. Credence was given to this possibility in a previous paper (Bond, 1951*b*), but the opinion has now been reached that it can be dismissed, both in respect of the present experiments with alder and the previous ones with *Myrica*, the reasons for this being as follows:

(*a*) In a culture jar containing several inoculated young plants those which nodulate will flourish, but any that fail to form nodules make little growth and behave just like the plants of control jars.

(*b*) If young uninoculated plants of *Myrica* are placed in the same jars as nodulated alder plants, the former make no growth.

(*c*) If the nodules are removed from an alder plant growing in nitrogen-free solution the plant quickly develops all the symptoms of nitrogen starvation.

No evidence emerges from the above for extra-nodular fixation, the products of which would be expected to become available to all the plants in a given jar. Item (*c*) points clearly to the conclusion that the fixation is dependent on the presence of nodules and that it probably occurs in the nodules, as is also supported by the relatively high percentage nitrogen content shown by the nodule tissues (p. 182). For similar reasons the same conclusion has been reached in respect of the earlier experiments with *Myrica*.

The alder is normally nodulated under field conditions, and there is no reason for doubting that fixation occurs under those conditions also. Some rough computations based mainly on the data reported in this paper suggest that the annual fixation by an alder tree of moderate size is likely to be of the order of 0·25 to 0·5 kg. nitrogen. According to information kindly provided by the Forestry Commission there are 24,500 acres of alder woods in Great Britain, a total which includes only woods of 5 acres and upwards and takes no account of narrow belts of riverside trees. It is clearly likely that the fixation by all these trees constitutes a quite significant contribution to the total biological fixation of nitrogen in this country.

Effect of pH on nodulation and growth

The results presented show that the infection of the plant and the initiation of nodules occur most freely over the pH range 5·4 to 7·0, and possibly best of all at 7·0. It is also shown that plants supplied with nitrate-nitrogen grow perfectly well at pH 3·3 or 4·2, making it clear that the failure of plants to nodulate at these levels (particularly the lower one) is due to a lower acid tolerance on the part of the nodule organism.

This new information may be of interest in ecology and forestry, as indicating conditions under which satisfactory establishment of alder may be expected. It may also be of some significance in connexion with the identity of the alder endophyte. The attack on this problem by the normal methods of cytological study of the infected cells of the nodule and of attempted isolation of the endophyte has led to divergent conclusions (Hawker and Fraymouth, 1951), and it is possible that increased knowledge of the physiological properties of the endophyte will provide a basis for more definite conclusions regarding its identity. Comparing the endophytes of alder and *Myrica* (Bond, 1951*b*), the latter shows somewhat greater acid tolerance since occasional plants formed nodules at pH 3·3. The minimal pH values for the nodulation of typical legumes are appreciably higher, as instanced by the finding of Jensen (1943) that red clover failed to nodulate below pH 4·4 and lucerne below 5·1.

The pH requirements for the subsequent growth of nodulated plants are lower than for the initiation of nodules, since the data show that the most favourable pH for growth and fixation of nitrogen is no higher than 5·4. Virtanen and Saastamoinen (1936), in respect of somewhat older plants than were used here, concluded that in sand culture nodulated alders grew better at pH 6 than at 5 or 7. It has been noted already that those authors had only a few plants at each pH, while differences in the conditions of the experiments may also have contributed to the rather small divergence in result.

Effect of aeration of culture solution

Virtanen and Saastamoinen (loc. cit.) also grew some plants in non-aerated water culture and observed that the nodules tended to develop near to the surface of the solution. This they thought indicated a relatively high oxygen requirement on the part of the nodules.

The present authors have shown that forced aeration of the culture solution is specifically beneficial to the development of nodulated plants, but caution is necessary in interpreting the results. The beneficial effect was shown at a very early stage in the experiment, since within a month from the commencement more nodules were present and growth was already superior in aerated jars. The oxygen data show that not until a considerably later stage did the respiration of the root systems suffice to lower the oxygen content to any marked extent. While it is possible that the multiplication of the nodule organism and the infection process are of highly aerobic nature, the alternative explanation seems more likely, namely, that the stirring effect of the air current assisted the dispersal of the organism over the newly forming parts of the root system and thus increased the number of nodules. That the formation of submerged nodules is not dependent on a high oxygen content is borne out by the previous observation that in non-aerated jars such nodules were produced when inoculation was effected at a relatively late stage.

It is highly likely that in the subsequent stages of the experiment the extra oxygen furnished by the forced aeration enabled the submerged nodules to function effectively. Table V shows that the relation of nodule dry weight to nitrogen fixed is similar in both aerated and non-aerated jars. But when submerged nodules are present without aeration, as in the second group of plants in the pH experiment, then effectiveness in fixation is lowered. Thus in Table II it is seen that fixation per unit dry weight of nodule tissue is considerably lower in the second group than in the first, the nodules in the latter being mostly near the surface.

The general implication of this experiment is that the nodule tissues have a higher oxygen requirement than that of the root tissues, since the oxygen supply in non-aerated jars of combined nitrogen plants appears to have been adequate for root function. It is intended to investigate this matter further by means of a technique giving greater control over oxygen supply, as used previously for legumes (Bond, 1951*a*).

Comparisons between legume and non-legumes symbioses

Using the data presented above for alder and previously for *Myrica* (Bond, 1951*b*) certain comparisons can be made with typical legumes (Table VII). All the data in the table refer to plants grown in nitrogen-free water culture under generally similar and favourable growth conditions. Those for legumes apply to plants associated with an 'effective' strain of the nodule organism (except in one instance) and grown from seed to a late flowering stage or for 10 weeks in the case of clover. The data for the perennial non-legumes apply to the first year of development.

TABLE VII

Comparison of Legumes and Non-legumes

Plant.	1 Mg. N fixed per plant.	2 Mg. N fixed per g. nodule dry matter formed.*	3 Nodule dry wt. as % of whole plant dry wt.
Field bean†	155	691	4·0
Soya bean	160	258	8·9
Soya bean‡	2	66	5·2
Field pea	178	539	5·3
Red clover	20	703	4·0
Alder	301	814	2·2
Myrica gale	34	420	6·6

* The method of calculating the figure in this column may be illustrated by taking the case of alder. Table II shows that at pH 5·4 (1st group) the formation of 0·37 g. nodule dry matter was accompanied by a fixation of 301 mg. nitrogen. Thus for the formation of 1 g. nodule dry matter the corresponding fixation would be $\frac{301}{0{\cdot}37} = 814$ mg.

† = *Vicia faba* L. ‡ With ineffective strain of nodule organism.

In legumes, assuming an 'effective' organism, the magnitude of the value in column 1 depends chiefly on the innate capacity of the host plant for photosynthesis and growth. That the value for alder exceeds all the legume values may be ascribed to the longer period (6 months) of vegetative growth, but implicit in this explanation is that the nitrogen-fixing system operates with comparable effectiveness to that in legumes. *Myrica gale* at the end of its first year is comparable in size with clover, and it will be seen that the fixation is of the same order.

Column 2 allows of a comparison of the effectiveness of the nodule tissues in fixation, the view being taken here that the nodule is the site of the fixation process (p. 184). With legumes wide variation is shown in the intrinsic activity of the nodules, depending on the particular strain of organism present. Relatively low activity is displayed when the nodules are occupied by an 'ineffective' strain, as instanced by Soya bean in the table. It is evident that the non-legume nodules achieve an effectiveness in fixation which is fully comparable with that of legumes associated with effective strains, the value for alder surpassing those for legumes.

Turning to column 3, the correlative influences limiting the development of nodule tissue are likely to be partly nutritive in nature and dependent on competition beween various parts of the plant body for materials required for growth. From the standpoint of the host plant the smaller the amount of nodule tissue, consistent with adequate fixation, the better, the drain on host metabolism being thereby minimized. In this sense the alder is the most efficient of the symbioses listed.

Summary

In agreement with the observations of previous investigators it is shown that nodule-bearing alder plants are able to grow vigorously in water culture without any external source of combined nitrogen. Evidence is advanced which indicates that the fixation of atmospheric nitrogen thus implied occurs within the nodulated plant and probably in the nodules. There is no reason to doubt that fixation is associated with alder trees in the field and that it is of ecological and general importance.

With artificial inoculation nodules appear at a similar stage in the development of the host plant as in legumes, and their formation occurs most freely over the pH range 5·4 to 7·0. Some nodulation is shown at pH 4·2 but none at 3·2, whereas non-nodulated plants supplied with nitrate-nitrogen grew well at these low pH levels, indicating that the nodule organism is unable to tolerate relatively acid conditions.

Subsequent to nodulation the growth of plants was better at pH 5·4 than at higher values, and was also satisfactory at pH 4·2. These statements refer to the first year of development of plants.

Forced aeration of water cultures markedly increased the growth of nodulated plants but had no such effect on non-nodulated plants supplied with combined nitrogen. The benefit is thought to have been due partly to the stirring effect of the air-stream, but also to the stimulatory influence of the increased oxygen supply on the functioning of the nodules, especially of the submerged ones. The nodules appear to have higher oxygen requirements than the roots.

It is shown that the fixation of nitrogen per plant by alder in its first season exceeds that of various legumes, and that fixation per unit dry weight of nodule tissue in alder and in *Myrica* is comparable with or greater than that in legumes.

Most of the experimental work reported in this paper was carried out by one of us (T. P. F.) during the tenure of a Carnegie Research Scholarship. The authors are indebted to Mr. D. N. McVean for providing some of the alder seed, and to Messrs. W. Anderson and J. MacArthur for photographical and technical assistance respectively.

Literature Cited

Bond, G., 1949: Root Nodules of Bog Myrtle or Sweet Gale (*Myrica gale* L.). Nature, clxiii. 730.

—— 1951*a*: Symbiosis of Leguminous Plants and Nodule Bacteria. IV. The Importance of the Oxygen Factor in Nodule Formation and Function. Ann. Bot., n.s., xv. 95–108.

—— 1951*b*: The Fixation of Nitrogen associated with the Root Nodules of *Myrica gale* L., with Special Reference to its pH Relation and Ecological Significance. Ibid., 447–59.

—— 1952: Some Features of Root Growth in Nodulated Plants of *Myrica gale* L. Ibid., 467–76.

Borm, L., 1931: Die Wurzelknöllchen von *Hippophaë rhamnoides* und *Alnus glutinosa*. Bot. Arch., xxxi. 441–88.

Fox, H. Munro, and Wingfield, C. A., 1938: A Portable Apparatus for the Determination of Oxygen Dissolved in a Small Volume of Water. Journ. Exp. Biol., xv. 437–45.

Hawker, L. E., and Fraymouth, J., 1951: A Re-investigation of the Root-Nodules of Species of *Elaeagnus*, *Hippophaë*, *Alnus*, and *Myrica*, with Special Reference to the Morphology and Life Histories of the Causative Organisms. Journ. Gen. Microbiol., v., 369–86.

Hiltner, L., 1896: Über die Bedeutung der Wurzelknöllchen von *Alnus glutinosa* für die Stickstoffernährung dieser Pflanze. Landw. Vers.-Sta., xlvi. 153–61.

Jensen, H. L., 1943: Nitrogen Fixation in Leguminous Plants. IV. The Influence of Reaction on the Formation of Root Nodules in *Medicago* and *Trifolium*. Proc. Linn. Soc. N.S.W., lxviii. 207–20.

Krebber, O., 1932: Untersuchungen über die Wurzelknöllchen der Erle. Archiv. f. Mikrobiol., iii. 588–608.

Möller, A., 1912: Ein neues Vegetationshaus und praktische Erprobung. Zeitschr. f. Forst- u. Jagdwesen, xliv. 527–38.

Nobbe, F., and Hiltner, L., 1904: Über das Stickstoffsaammlungsvermögen der Erlen und Elaeagnaceen. Naturwiss. Zeitschr. f. Land- u. Forstwirtschaft, ii. 366–9.

Plotho, O. V., 1941: Die Synthese der Knöllchen an den Wurzeln der Erle. Archiv. f. Mikrobiol., xii. 1–18.

Roberg, M., 1934: Weitere Untersuchungen über die Stickstoffernährung der Erle. Ber. deutsch. bot. Ges., lii. 54–64.

Virtanen, A. I., and Saastamoinen, S., 1936: Untersuchungen über die Stickstoffbindung bei der Erle. Biochem. Zeitschr., cclxxxiv. 72–85.

EXPLANATION OF PLATE

Illustrating T. P. Ferguson's and G. Bond's article on 'Observations on the Formation and Function of the Root Nodules of *Alnus glutinosa* (L.) Gaertn.'

Fig. 1. Plant after 14 weeks' growth in water culture, showing nodules localized near base of stem. ($\times \frac{1}{4}$.)

Fig. 2. Plant grown for 19 weeks in water culture, showing scattered, submerged nodules. ($\times \frac{1}{4}$.)

Fig. 3. Typical nodulated plants from the 1951 experiment (2nd group) after 21 weeks' growth at pH from left to right: 4·2, 5·4, 6·3, 7·0. ($\times \frac{1}{11}$.)

Fig. 4. Plants grown for 30 weeks in water culture without combined nitrogen at pH 7·0; on the left a control plant without nodules, on the right a nodulated plant, from the 1951 experiment (1st group). ($\times \frac{1}{12}$.)

Fig. 5. On the left nodulated plants in nitrogen-free solution (from 1951 experiment, 1st group), on the right non-nodulated plants supplied with nitrate-nitrogen, both after 21 weeks' growth at pH 5·4. ($\times \frac{1}{11}$.)

Fig. 6. A nodulated plant transplanted as a seedling into nitrogen-free water culture in May 1950 and photographed September 1951. pH maintained at 5 approximately. ($\times \frac{1}{14}$.)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An Apparatus for Automatic Recording of Losses of Water from Potted Plants, Soil, and Water Pans

BY

KHAZAN SINGH

With Plate IV and one Text-figure

Abstract

An apparatus for automatic recording of losses of water from potted plants, soil, &c., has been described. It is simple, and sufficiently robust for use in the open. Results on the daily march of transpiration of wheat in the northern plains of India have been given to demonstrate the working of the apparatus.

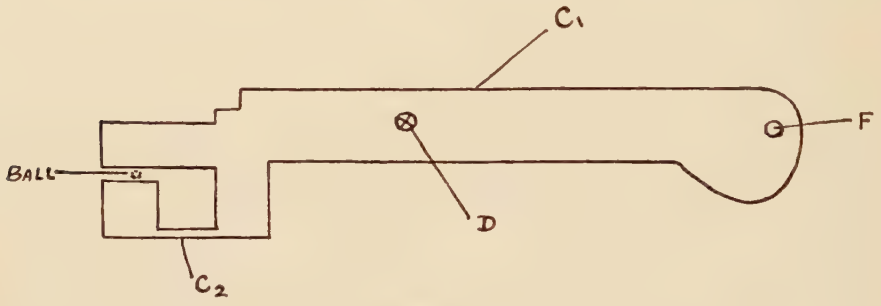
AUTOMATIC recording of water loss becomes essential in experiments connected with a study of the daily march of transpiration and evaporation. Briggs and Shantz (1915), Blackman and Paine (1914), and Henderson (1926), among other workers, employed automatic transpiration scales for recording the loss of water from potted plants and water pans. The automatic scales used by these investigators (except Henderson) have been of an elaborate nature and costly. An apparatus was designed which could be simple and cheap and could be attached to an ordinary steel yard.

Description of the Apparatus

The working of the apparatus is based on an electromagnetic device by which balls are released from time to time, disturbing a lever of a horizontal weighing machine. A long spiral glass tube, Pl. IV, Fig. 2, A_1, A_2, holds metallic balls B, each 1 g. in weight. The last portion of this tube A_3, consists of a $4 \times \frac{1}{2}$ in. brass tube. It lies slanting on one side of a brass box H, $6 \times 4 \times 2$ in., and is fixed at each end by soldering. The spiral tube with the ball reservoir cup A is joined to this tube to make a continuous array of balls inside the entire length of the tube. Two half-way slots are made across the brass tube, one on the lower and one on the upper side of the tube and $\frac{1}{8}$ in. apart from one another (equal to the diameter of the ball). A lever C, with its side protrusion C_2, is made of the given shape (Text-fig. 1) from a $5 \times 1\frac{1}{8} \times \frac{3}{32}$ in. brass strip and is pivoted on a U-shaped stand D by means of two fine-pointed screws E so that it rests centrally balanced on the stand. The U-shaped stand is fixed to the floor of the brass box, $1\frac{1}{2}$ in. away and in line with the upper slot of the tube. The end of the main part of the lever fits into the upper slot, while the protrusion goes under the lower slot in its resting position. The lever blocks the ball tube A_3 in this position (see Pl. IV, Fig. 2).

[Annals of Botany, N.S. Vol. XVII, No. 65, January, 1953.]

The lever of the horizontal weighing machine L (Pl. IV, Fig. 3) is counterbalanced by putting weights M equal to the weight of the object O. When it is disturbed by the loss in weight of the object, the two electric contacts K touch each other and the circuit is closed. (The end of the lever of the machine acts as one contact.) The magnet G is energized by the accumulator and pulls the lever C. The end of the lever blocking the ball tube and the protrusion are simultaneously raised, so that now the latter blocks the ball tube against the ball next to the one which is released and drops into the bowl N. The lever of the weighing machine is again disturbed and this opens the circuit and



TEXT-FIG. 1

releases the lever C. The pen F, which is fixed to the other end of the lever C, makes a downward stroke on the clockwise revolving drum J as soon as the lever is pulled by the magnet. It returns to its position after the lever is released. The record consists of straight lines on a time scale, as shown in Pl. IV, Fig. 3. From these the loss in weight of the object at a particular time can be assessed.

EXPERIMENTAL RESULTS

One-day results on the daily march of transpiration of wheat (Var. P. 52) out of doors in northern plains of India give a measure of the working of the apparatus. A potted wheat plant was placed inside an aluminium container with two split tops and sealed air-tight with paraffin-wax mixture. It was then placed on the platform of the weighing machine. The balls of the bowl were counted after every 3 hours, and the potted plant was also weighed on a separate counterpoised balance to check on readings of the graph. The losses of weight at different times of day for the preceding 3 hours were:

Time in hours	6	9	12	15	18	21	24	0–6
Loss (g.)	11	11	25	20	6	2	1	3

These figures give an adequate idea of the scope of the method.

Transpiration under these conditions reaches its maximum by 12 noon and falls in the afternoon. Further, night transpiration is only 9 per cent. of the total for 24 hours. Briggs and Shantz (1916) found the maximum transpiration of cereals was reached between 2.00 and 4.00 p.m.

ACKNOWLEDGEMENTS

My thanks are due to Mr. A. G. Pollard, Head of the Department of Agricultural Chemistry, Imperial College, London, S.W. 7, for his suggestions during the preparation of the text. As a State Scholar I am indebted to the Indian Government for their support and help.

LITERATURE CITED

BLACKMAN, V. H., and PAINE, S. G., 1914: Ann. Bot. N.S., xxviii. 109–13.
BRIGGS, L. J., and SHANTZ, H. L., 1915: J. Agri. Res. v. 117–33.
—— —— 1916: ibid., v. 583–649.
HENDERSON, F. Y., 1926: Ann. Bot. N.S., xl. 507–33.

EXPLANATION OF PLATE

Details of apparatus for recording automatically loss of water from potted plants.

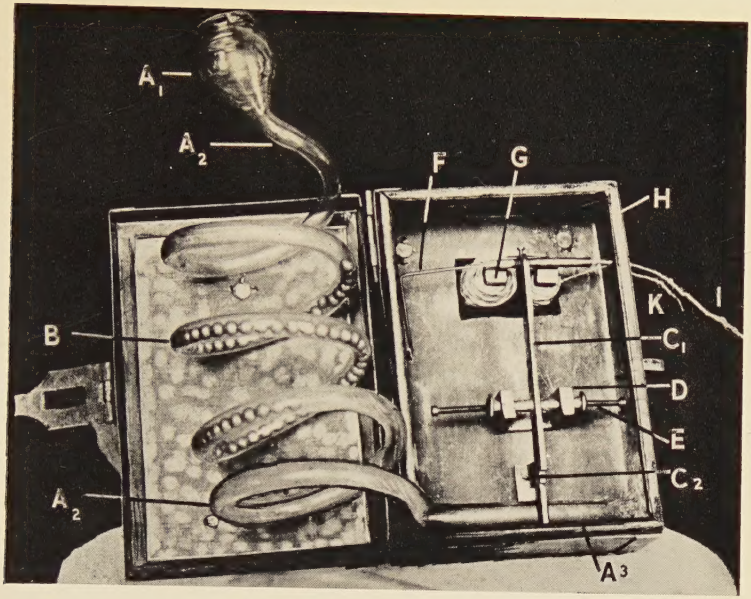


FIG. 2. Apparatus assembly

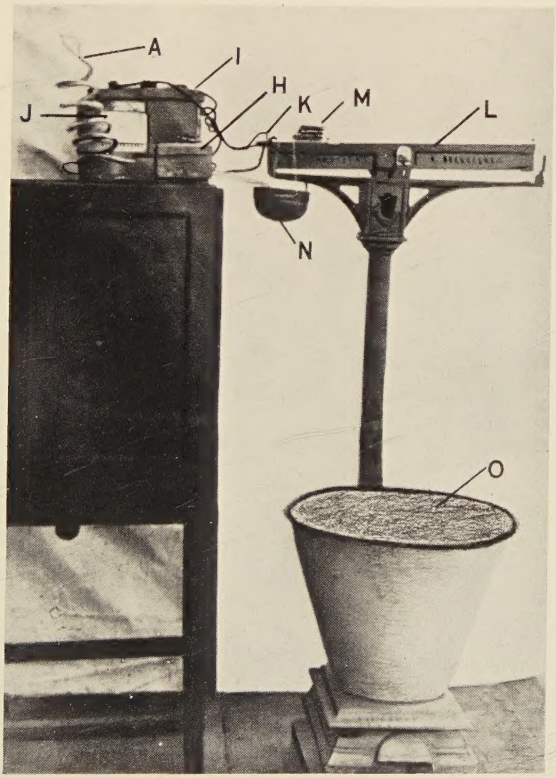


FIG. 3. Arrangement of the apparatus with the weighing machine